Drexel University

Annual Progress Report: 2014 Formula Grant

Reporting Period

January 1, 2015 – June 30, 2015

Formula Grant Overview

The Drexel University received $1,079,197 in formula funds for the grant award period January 1, 2015 through December 31, 2015. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Population Pharmacokinetics of Cefazolin on Cardiopulmonary Bypass in Neonatal and Pediatric Patients – The purpose of this study is to determine the pharmacokinetics of cefazolin used in cardiopulmonary bypass (CPB) circuits to characterize the pharmacokinetic changes related to the CPB circuit. The pharmacokinetics will be evaluated to determine whether a dosing equation or constant can be derived to apply to initial dosing regimens to account for the pharmacokinetic alterations with CPB circuits. Population pharmacokinetic estimates will also be derived and contemporary pharmacokinetic/pharmacodynamics analysis will be conducted to determine an optimal dosing regimen for patients on CPB.

Anticipated Duration of Project

1/1/2015 – 12/31/2016

Project Overview

The purpose of this study is to determine the pharmacokinetics of cefazolin in cardiopulmonary bypass (CPB) circuits and characterize the pharmacokinetic changes. The specific aims are as follows; 1) determine the pharmacokinetics of cefazolin in cardiac surgery patients on CPB, 2) determine population pharmacokinetic estimates for children receiving cefazolin on CPB, 3) conduct pharmacodynamic analysis of current cefazolin dosing recommendations for patients on CPB, 4) determine optimal dosing regimen of cefazolin for patients on CPB. This study will be a prospective pharmacokinetic analysis. Ten patients who would receive cefazolin during their CPB run stay in each of the following age groups (birth-3 months, >3-6 months, > 6 months-3 years, > 3 years to 11 years, and 12-16 years) will be enrolled. Cefazolin will be given as a bolus over 5 minutes and serial serum concentration levels will be measured pre and post oxygenator at different time points during the dosing interval. Individual pharmacokinetic parameters will be

Note: This project is funded, in part, under a grant with the Pennsylvania Department of Health. The Department specifically disclaims responsibility for any analyses, interpretations or conclusions.

determined which will then be used to derive population pharmacokinetic estimates. The population pharmacokinetic estimates will then be used to determine an optimal dose and interval to provide the pharmacodynamic endpoint of interest (i.e., appropriate concentration above the minimum inhibitory concentration (MIC) for the duration of the procedure). The effect of specific patient characteristics (e.g., age, body weight, BMI, etc.) on parameter estimates will be explored. Based on the cefazolin pharmacokinetic profile and the MIC of the potential causative pathogens, we will determine the cefazolin exposure needed. These data collectively will be utilized to identify an optimal cefazolin dose for children undergoing cardiac surgery on CPB. The study population will include 50 patients, 10 per age group, undergoing cardiac surgery requiring CPB. The age range of birth-16 years of age will allow for pharmacokinetic variability that may be due to changes in age, body weight, or other physical characteristics. Participants will be enrolled in equal numbers (10 per group) to the following age subgroups so that adequate age populations are represented in the final pharmacokinetic analyses: birth-3 months, >3-6 months, > 6 months-3 years, > 3 years to 11 years, and 12-16 years.

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Expected Research Outcomes and Benefits

The expected outcome will be to derive population pharmacokinetic estimates to determine an appropriate dosing regimen for patients receiving cardiac surgery on CPB. Currently, there are many different dosing strategies and regimens used in clinical practice and it is expected that the results of this project, using the population estimates, will provide insight and guidance on a dosing regimen that provides for appropriate drug exposures.

Summary of Research Completed

Specfic Aim 1 Progress:
During this reporting period, SCHC has screened 18 eligible patients for enrollment and subsequently enrolled 17 patients in the study and obtained samples and data for each of the 17 patients. The data use agreement is under negotiation between the Alfred I. duPont Hospital for Children (AIDHC) and Drexel University/St. Christopher's Hospital for Children.

Subjects were recruited during routine cardiology and cardiothoracic clinic visits when it was determined that they will be admitted for cardiac surgery that would require bypass.

Patients received cefazolin during their CPB run stay for each of the following age groups (birth-3 months, >3-6 months, > 6 months-3 years, > 3 years to 11 years, and 12-16 years) were enrolled. Cefazolin was given as a bolus over 5 minutes and serial serum concentration levels were measured pre and post oxygenator at different time points during the dosing interval. Individual pharmacokinetic parameters were determined which will then be used to derive a population pharmacokinetic estimate. The population pharmacokinetic estimates will then be used to determine an optimal dosing and interval to provide the pharmacodynamic endpoint of interest (i.e. appropriate concentration above the minimum inhibitory concentration for the duration of the procedure).

Cefazolin was administered as a bolus infusion over of a period of < 5 minutes. The first blood samples were collected 10 minutes after the bolus. Subsequent blood samples were obtained every 20 minutes until the patient was off bypass. Two samples were collected at each time point, one that was pre-oxygenator and one that was post-oxygenator. At the time of removal of the patient from bypass, a serum concentration from the patient was obtained in addition to a sample from the bypass machine. A minimum of 2 mL (~ ½ teaspoon), with a maximum of 10 mL (2 teaspoons), could be required for each sample, with 5 mL being the ideal sample volume (1 teaspoon).

After all data are collected, a population analysis will be conducted to describe the pharmacokinetics of cefazolin and analyze the effect of specific patient characteristics (e.g., age, body weight, BMI, etc.) on parameter estimates. Based on the cefazolin pharmacokinetic profile and the minimum inhibitory concentration of the potential causative pathogens, we will determine the cefazolin exposure for each participant. These data collectively will be utilized to identify an optimal cefazolin dose for children undergoing cardiac surgery on CPB.

Data Collection

Patient data that was collected including gestational age, age, gender, delivery and resuscitation details, blood urea nitrogen (BUN), creatinine levels, urine output, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), fibrinogen, prothrombin time (PT), and partial prothrombin time (PTT). Further details regarding the type of surgery, length of bypass, cross-clamp time, dosage of cefazolin, the priming technique, volume used for priming, surgery duration, and any other drugs given during the surgery that may have an impact on the cefazolin serum concentrations. After the patient's chart was identified and data collection was complete, all patient identifiers were removed and the data was recorded in a password protected database with the same random number as that assigned to the patient's blood samples

Sample Processing

Blood samples were allowed to clot for 5 minutes and then removed from ice after 5 minutes. The samples were then allowed to stand until clotting was completed, if required. Serum was separated as soon as possible after clotting. Equal amounts of serum were transferred into two appropriately labeled white capped cryovials. [Note. One cryovial per time point is labeled as a

BACKUP and is held at SCHC in case of shipping errors]. Samples were stored at -80° C until ready for shipment. Samples are batched and subsequently shipped to an outside third party for analysis via liquid chromatography tandem mass spectroscopy (LC-MS/MS).

Specfic Aim 2 Progress:
No progress was achieved during the current reporting period for this aim.

Specfic Aim 3 Progress:
No progress was achieved during the current reporting period for this aim.

Specfic Aim 4 Progress:
No progress was achieved during the current reporting period for this aim.

Table 1. Enrollment numbers broken down by age per site:

Age	SCHC	NYU	duPont	Age totals
Birth to 3 mths	1			1
>3-6 mths	5			5
>6 mths- 3 yrs	8			8
>3-11 yrs	2			2
12-16 yrs	1			1
Site totals	17	0	0	17

Research Project 2: Project Title and Purpose

Early Exercise Intervention Prevents Spinal Cord Injury-induced Neuropathic Pain Development by Modulating the Levels of Pro- and Anti-inflammatory Cytokines – The purpose of this experiment is to determine whether exercise prevents pain development after spinal cord injury (SCI) by modulating the post-injury inflammatory response throughout the spinal cord and brain as well as systemically. By examining the inflammatory response in the brain and spinal cord, we will understand how inflammation affects pain signaling. Detection of changes in systemic inflammatory response after SCI and/or exercise over time will identify potential biomarkers or predictors of pain development that could be directly translated to clinical SCI. These biomarkers would also inform on potential pharmacologic targets, as well as the effectiveness of the exercise, and lead to refinement of the rehabilitation protocol.

Anticipated Duration of Project

1/1/2015 – 12/31/2016

Project Overview

Glia can be activated by immune and neuronal cytokines and may play a critical role in the onset and modulation of chronic neuropathic pain after spinal cord injury (SCI). Exercise is widely used in the clinical SCI population, and we showed that early administration of exercise prevents neuropathic pain development in experimental SCI. We will test the hypothesis that exercise prevents the development of SCI-induced neuropathic pain by modulating the systemic and local inflammatory response.

AIM 1: Determine whether unilateral cervical SCI elevates levels of pro-inflammatory cytokines in circulation and along the sensory neuroaxis that correlates to the onset and persistence of forelimb neuropathic pain. This experiment will provide the basis for assessing the direct effect of pro-inflammatory cytokines on the development of SCI-induced pain.
AIM 2: Determine whether exercise prevents pain development after SCI by modulating the degree of the systemic and local inflammatory response. This experiment will show whether the prevention of SCI-induced pain by exercise correlates with a reduction in the inflammatory response or a change in the ratio of pro- to anti-inflammatory cytokines.

For both aims, we will use a rat unilateral C5 spinal cord contusion model where 40% of injured rats develop persistent neuropathic pain. A subset of SCI rats will receive exercise training in automated running wheels 20min/day 5d/week for 5 weeks starting at 5 days post injury (dpi).The levels of circulating cytokines (CCL2, TNFa, IL-1b, IL-6, IL-12, IL-4, IL-10) at baseline, 7, 14, 28 and 35 dpi will be measured using microelectrophoresis technology (www.proteinsimple.com). We will correlate the levels of each cytokine in circulation to the induction and persistence of neuropathic pain. At 35 dpi, rats will be sacrificed, and the levels of these cytokines within the DRG, dorsal horn, and ventroposterolateral nucleus of the thalamus (VPL) will be measured. Additionally, the microglia/macrophage response will be determined via immunohistochemistry (tomato lectin, Iba-1, CCR2, ED-1, ArginaseI, CD206) in the DRG, spinal cord and VPL of SCI rats with and without pain.

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Expected Research Outcomes and Benefits

We expect that rats with spinal cord injury (SCI)-induced pain to have elevated circulating pro-inflammatory cytokines as well as elevated levels within the dorsal root ganglia (DRG), dorsal horn of the spinal cord and/or ventroposterolateral nucleus of the thalamus (VPL) compared to uninjured rats and SCI rats that do not develop pain. As SCI causes a robust inflammatory response along the sensory neuroaxis, we would be remiss to expect exercise to normalize the levels of pro-inflammatory cytokines circulating in the blood. Rather, we expect that exercise intervention would either prevent the elevation of pro-inflammatory cytokines to levels similar to unexercised SCI rats without pain. It is entirely possible that exercise does not reduce the levels of pro-inflammatory cytokines (Tumor necrosis factor (TNF)-α, Interleukin (IL)-1β, IL-6, IL-12) but rather that it increases the levels of anti-inflammatory cytokines (IL-4, IL-10) that are circulating or present in DRG, spinal cord and VPL. Change in the ratio of pro- to anti-inflammatory cytokines may have profound impact on the development of chronic SCI-induced pain. We also expect that there will be immunohistochemical evidence of inflammation in the DRG, spinal cord and VPL tissue. Inflammation will be examined immunohistochemically in the DRG, spinal cord and VPL by staining the tissue for microglia. Importantly, microglia can express markers that indicate whether they are acting in a pro-inflammatory (CCR2+) or anti-inflammatory (Arginase+, CD206+) manner. This research will benefit the scientific and clinical community by providing: 1) potential biomarkers of chronic pain development, 2) inflammatory targets for future pharmacologic intervention, and 3) information regarding a mechanism for by which exercise is working to modulate the development of chronic pain after SCI.

Summary of Research Completed

Specific Aim 1 Progress:
For in this aim, we are testing the hypothesis that in animals that develop neuropathic pain as a result of incomplete cervical spinal cord injury exhibit a distinct "pain-specific" inflammatory response. To test this hypothesis, adult, female rats received incomplete cervical SCI and rats were tested for the development of neuropathic pain with the von Frey and an operant mechanical conflict avoidance test for changes in tactile sensation, and the Hargreaves' test for changes in thermal sensation. Additionally, blood was collected weekly to assess changes in circulating inflammatory and anti-inflammatory cytokines. Four weeks post SCI, rats were sacrificed and sensory centers in the nervous system were harvested for protein analysis via ELISA or immunohistochemistry. All experimental work was approved by the Institutional Animal Care and Use Committee of Drexel University College of Medicine. We have completed three quarters of the animal experiments for this aim at this time (14/20 rats have been spinal cord injured, undergone sensory behavior testing, and their tissue has been harvested; 5/10 naïve rats have undergone sensory behavioral testing and their tissue has been harvested). Detailed descriptions of our current findings for Aim 1 are presented below.

Specific Aim 2 Progress:
For in this aim, we are testing the hypothesis that exercise prevents pain development after SCI by modulating the systemic and local inflammatory response. To test this hypothesis, adult female rats received incomplete cervical spinal cord injury as described in Aim 1. Rats began daily exercise training 5 days post injury at 5 m/min, 20 min/day, 5 days/week in an automated wheel walking system (Lafayette Instruments, Lafayette, IN). Wheel rotation increased according to the capabilities of the fore limb up to 14 m/min is reached (speed of step training on a treadmill for uninjured rats). All behavioral assessments, blood collection and tissue harvest were conducted as described in Aim 1. We have completed three quarters of the animal experiments for this aim at this time (16/20 rats have been spinal cord injured, undergone sensory behavior testing, and their tissue has been harvested; 5/10 naïve rats have undergone sensory behavioral testing and their tissue has been harvested). Detailed descriptions of our current findings for Aim 2 are presented below.

DETAILED PROGRESS (Aims 1 and 2)

Analysis of Spinal Cord Lesions: For each spinal cord injury, we use a computer driven piston that rapidly displaces the spinal cord at a set force. The computer collects data on the force and displacement parameters. Figure 1 show the actual contusive force of the piston when it impacts the spinal cord (A) and the displacement of the spinal cord at maximum impact (B). Importantly, these two parameters were similar for both the SCI group and the SCI group that received exercise.

Behavioral Results for Aim 1 & 2: Animals were evaluated for tactile and thermal nociception prior to spinal cord injury and weekly post injury. Tactile sensation was measured using the von Frey test (Stoelting). The test was performed by applying a series of graded monofilaments to the paws of the rat. A positive response occurred when the rat withdrew its paw in response to monofilament application. Normal rats withdrawal their forepaws to a tactile stimulation of ~18 grams of force (Figure 2A, B, dashed line). We found that unexercised SCI rats either developed bilateral persistent tactile hypersensitivity in the forepaws that persists chronically or retained normal sensation (Figure 2A, B). Importantly, we found as expected that roughly 40% of the SCI rats become hypersensitive to tactile stimuli. Exercise for 20 minutes a day on automated running wheels that was initiated at 5 days post spinal cord injury prevented the development of tactile hypersensitivity in the forepaws (Figure 2A, B). Interestingly, while spinal cord injury generated two distinct behavioral cohorts, forepaw sensitivity to noxious thermal stimuli did not differ between any of the groups (Figure 2C, D). Thermal sensation was measured using the Hargreaves' radiant heat test. The test is performed by applying an infrared beam of light to the palmar surface of the paw and recording the latency or time until paw withdrawal. Normal rats withdrawal their forepaws after a ~10 second application of radiant heat. Thermal hypersensitivity occurs when the paw withdrawal latency decreases. After injury, rats show a trend toward a decrease in the latency to paw withdrawal.

Progress on Protein Analysis Aims 1 & 2: Since we plan to analyze at least 10 mediators of the inflammatory response, we are currently determining the best method to analyze our samples. Serum and tissue samples from the rat are relatively small, and quantities are limited. Initially,

we proposed to use microelectrophoresis technology to quantify the amount of these cytokines in our samples, but troubleshooting of the system revealed that the Protein Simple technology available was not sensitive enough to detect the concentrations of pro- and anti- inflammatory molecules that are present in spinal cord, dorsal root ganglia, thalamic brain regions and serum. We have recently sent pilot samples for quantification using an ELISA Multiplex technique at Eve Technologies. Additionally, we sectioned a subset of spinal cord, dorsal root ganglia and brain tissue of exercised and unexercised SCI rats. These tissues will then be analyzed for immunocytochemical markers of microglial and macrophage activation (cells that are a major part of the inflammatory response).

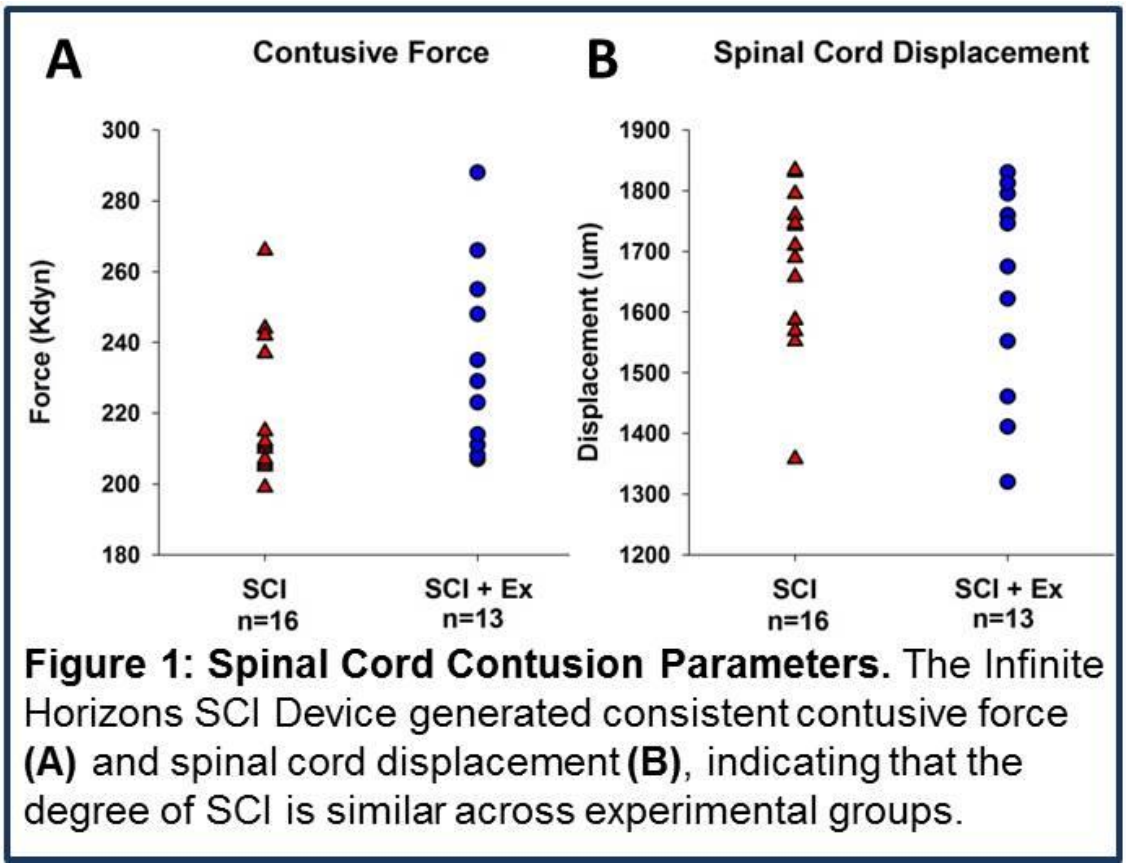

Figure 1: Spinal Cord Contusion Parameters. The Infinite Horizons SCI Device generated consistent contusive force **(A)** and spinal cord displacement **(B)**, indicating that the degree of SCI is similar across experimental groups.

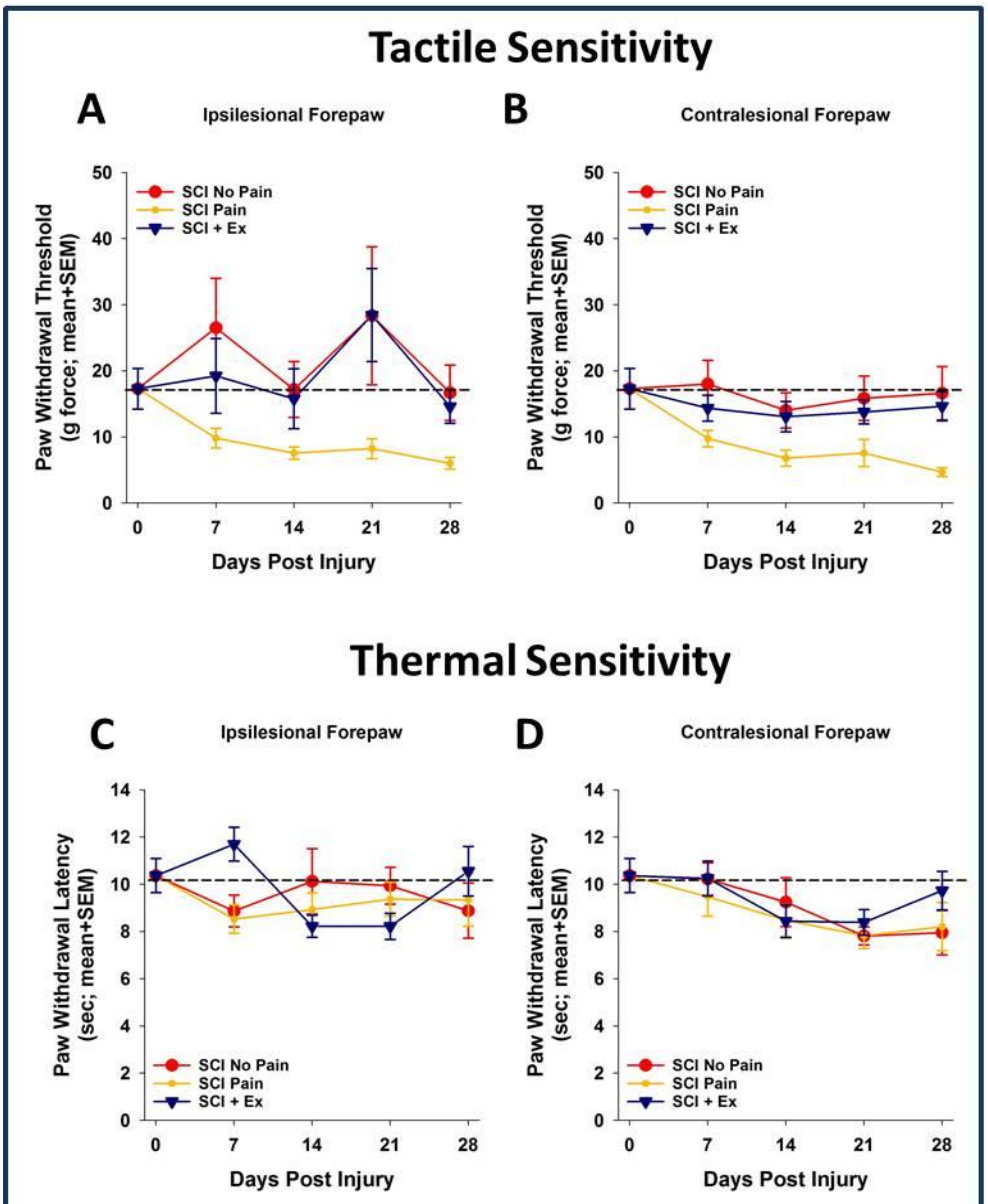

Figure 2: Neuropathic pain behavior after Unilateral C5 SCI. Rats with unilateral C5 contusion can be separated into two distinct groups after SCI, those with a >50% reduction in tactile sensory threhsold of the ipsilesional **(A)** and contralesional **(B)** forepaws (SCI Pain, yellow) and those which retain normal tactile sensory threhsolds (SCI No Pain, red). Exercise intervention beginning at 5 days post injury prevents tactile hypersensitivity (SCI + Ex, blue). Importantly, though some rats develop tactile allodynia and some do not, all rats with SCI have a similar paw withdrawal response to noxious heat **(C and D)**. *Dashed line indicates normal values.*

Research Project 3: Project Title and Purpose

Targeting Microtubule-severing ATPases in Glioblastoma – The research study will be undertaken to discover new knowledge leading to new treatment approaches in glioblastoma (GBM), a highly aggressive brain tumor that does not have a cure. High spastin expression correlates with increasing grade of malignancy and aggressive tumor behavior. The outlined approach will allow us to determine the therapeutic relevance of spastin in glioblastoma *in vivo* and investigate the mechanistic basis of spastin in drug chemosensitivity *in vitro*. This study has the potential of revealing a new drug target to improve the long-term survival of patients diagnosed with GBM.

Anticipated Duration of Project

1/1/2015 – 6/30/2016

Project Overview

In cancer cells microtubules (MTs) undergo complex changes involving a host of MT-related proteins, including MT-severing enzymes. We have previously shown that increased expression of the MT-severing protein spastin may define cancer cells with aggressive/invasive behavior in human glioblastoma (GBM) and that spastin depletion substantially inhibits cell motility of GBM cells *in vitro*. We propose to utilize this base of knowledge to test whether targeting spastin can block intra-cerebral tumor spread *in vivo* using an orthotopic human GBM model in mice thus establishing MT-severing proteins as potential therapeutic targets in brain cancer.

The objectives of this study are to elucidate the mechanisms by which abnormally expressed MT proteins contribute to aggressive tumor behavior and exploit them as potential therapeutic targets in GBM. The aims of this study are (a) to establish, for the first time at Drexel University, an orthotopic xenograft brain tumor model by which we will determine the effects of spastin silencing on tumor growth and spread *in vivo* using human GBM cell line-derived orthotopic xenografts transplanted into the brains of immunodeficient mice (Specific Aim 1) and (b) identify potential therapeutic synergy between spastin depletion and current GBM treatments *in vitro* (Specific Aim 2).

To determine whether spastin affects tumor cell growth and invasion *in vivo*, this protein will be depleted using stable lentiviral shRNA plasmids against spastin in human GBM cell lines. Tumor cells will be then surgically implanted into the brains of mice and growth and invasion in orthotopic xenografts will be closely monitored *in vivo* and *ex vivo*.

To identify potential synergy between spastin depletion and current GBM therapies *in vitro, w*e will systematically characterize the antineoplastic effects of spastin depletion in combination with currently used principal therapies including radiotherapy and chemotherapy (temozolomide and cisplatin). We will perform also a screen using the 114 currently FDA approved cancer drugs to identify additional FDA-approved cancer drugs that can synergize with spastin depletion to exert an abrogating effect on the growth and migration of GBM cells.

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Expected Research Outcomes and Benefits

Glioblastoma (GBM) is a highly aggressive brain tumor that is refractory to currently used treatment modalities and has a dismal prognosis. In view of that, innovative approaches to understand the biology and treatment of this neoplastic disease are urgently needed. High spastin expression correlates with increasing grade of malignancy and aggressive tumor behavior. The research study will be undertaken to discover new knowledge leading to new treatment approaches in GBM. The outlined approach will allow us to determine the therapeutic relevance of spastin in GBM *in vivo* and investigate the mechanistic basis of spastin in drug chemosensitivity *in vitro*. This study has the potential to increase our understanding of GBM biology and uncover a new drug target to improve the overall survival of patients. Although MT targeting has been a time-honored approach in cancer therapeutics, most currently used MT-binding drugs are aimed at mitotic spindle disruption and bind indiscriminately to αβ-tubulin heterodimers. The novel and innovative aspect of this proposal rests on the selective targeting of MT-regulating proteins, such as MT-severing enzymes, which have emergent roles in cancer behaviors, such as tumor cell migration and invasion. This study will provide a strong rationale for identifying and testing small molecules that target MT-severing enzymes, such as spastin, as potential therapies for GBM. Furthermore, we expect to identify currently used cancer therapies that will synergize with spastin depletion in human GBM cell lines *in vitro* and expect that spastin depletion may also synergize with treatment with other emerging FDA-approved cancer drugs. Collectively, the results will provide a strong rationale for discovering spastin-selective inhibitors in future experiments and determining mechanistically how this synergism occurs, as well as validating synergism using preclinical animal models.

Summary of Research Completed

Specific Aim 1 Progress:
We have begun to identify small hairpin RNA (shRNA) sequences that induce the most robust knockdown in cells from human GBM cell lines U87MG and T98G. We have identified at least three separate shRNAs that reduce spastin expression in GBM cells (Figure 1). Importantly, all three shRNAs identified have been shown to reduce expression of both full-length spastin as well as a short-form spastin variant (Figure 1).

We have generated T98G and U87MG human GBM cells stably expressing luciferase (Figure 2) that will be used to monitor tumor growth and spread using an animal imaging system.

We have received approval for animal protocol from Institutional Animal Care and Use Committee (IACUC) and have begun major animal experiments described in Aim 1 to test the role of spastin depletion on glioblastoma growth and spread *in vivo*. We are currently in the process of characterizing the effect of spastin depletion on tumor cell growth and invasion in vivo using an orthotopic xenograft mouse model.

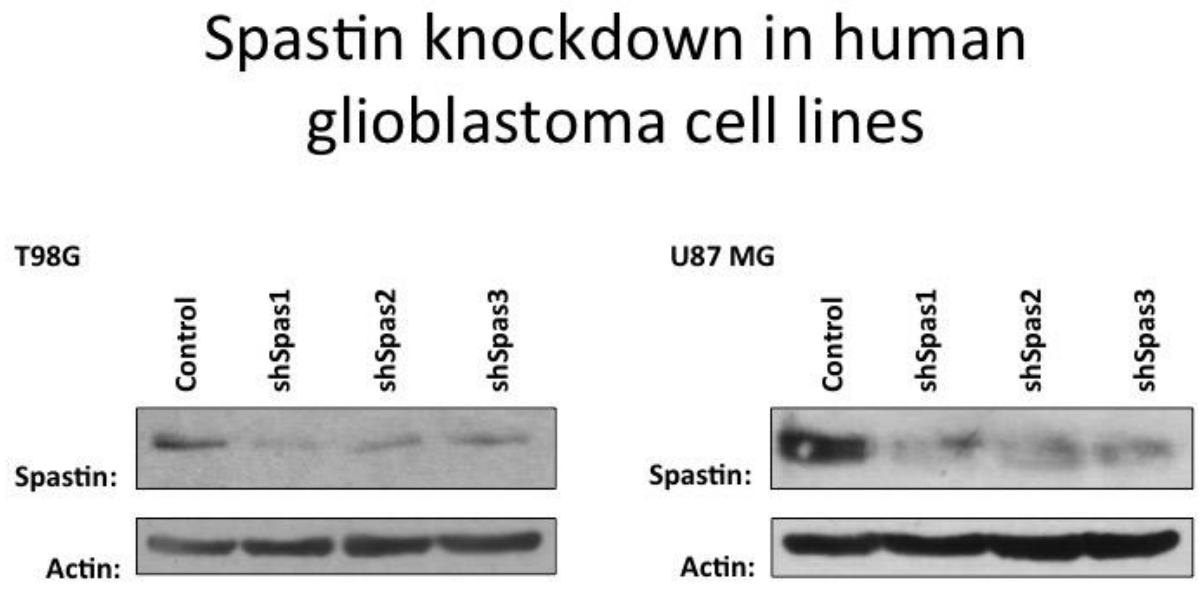

Figure 1. Spastin knockdown in glioblastoma cells. T98G and U87MG cells were stably infected with control or spastin shRNA. Lysates were collected and analyzed by western blotting with indicated antibodies.

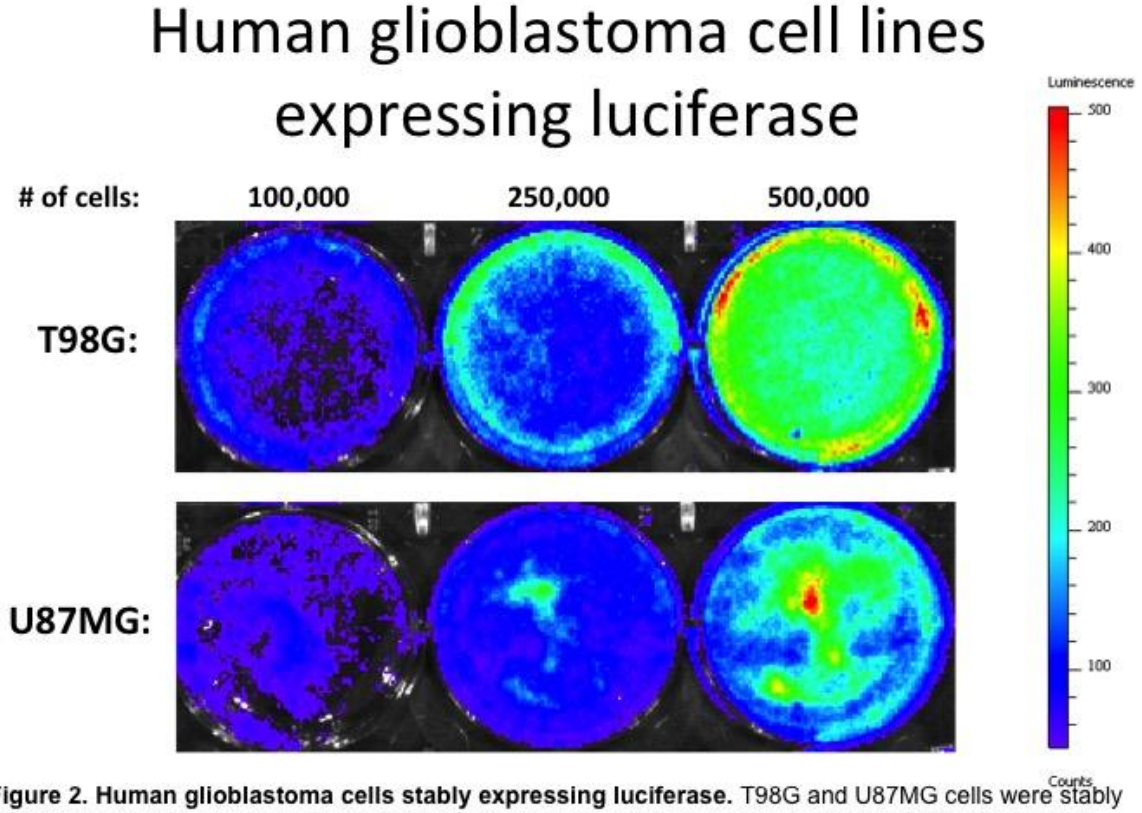

Figure 2. Human glioblastoma cells stably expressing luciferase. T98G and U87MG cells were stably infected with luciferase. Indicated number of cells were plated and analyzed using IVIS imaging system.

Specific Aim Progress:

Having successfully achieved knockdown of spastin in cells from GBM cell lines U87MG and T98G, we have begun experiments detailed in Aim 2 to examine whether chemotherapeutic agents used to treat patients with GBM can synergize with spastin depletion *in vitro*.

Research Project 4: Project Title and Purpose

Non-Thermal Plasma as an Effective DNA Vaccine Adjuvant – Dielectric barrier discharge (DBD) plasma, which is a form of non-thermal plasma (NTP), is generated by applying high voltage to an electrode encased in a dielectric material. This project will test the hypothesis that DBD plasma can be used to augment the delivery of a DNA-based vaccine. The project will provide proof-of-concept results and a solid basis for a more comprehensive research plan to be submitted as an NIH grant application. The ultimate goal of this work will be the development of a novel and non-invasive method for improving the delivery and increasing the efficacy of DNA-based vaccines effective against important human pathogens.

Anticipated Duration of Project

1/1/2015 – 6/30/2016

Project Overview

Dielectric barrier discharge (DBD) plasma, which is a form of non-thermal (NTP) plasma, is generated by applying high voltage to an electrode encased in a dielectric material. Non-thermal, atmospheric plasma has been shown *in vitro* and *in vivo* to have antibacterial, antifungal, and antiviral activities, as well as wound healing properties and cancer therapy potential. Although the mechanisms responsible for the effects of NTP are not completely understood, reactive oxygen species (ROS) have been shown to mediate the effects of NTP exposure. NTP has also been investigated as a means of delivering DNA vaccine constructs. In mouse model studies, the application of helium-based NTP resulted in enhanced delivery of an intradermally administered DNA vaccine construct, as indicated by increased antigen-specific antibody titers and interferon-γ-producing cells. These studies, however, did not address either the mechanism of action underlying enhanced vaccine expression or NTP safety. Investigations outlined in this project will test the hypothesis that DBD plasma can be used to augment the delivery of DNA vaccine expression constructs. DBD plasma has several advantages over the helium-based plasma source used in previous studies, including gas-free generation of plasma, more uniform application, and fine controls over plasma generation and delivery parameters. The specific aims of this project are to (1) examine DBD plasma-dependent changes in epithelial barrier function, epithelium-induced inflammation, immune cell recruitment, and DNA reporter uptake and expression, and (2) demonstrate antigen-specific responses augmented by DBD plasma application in a mouse model of DNA vaccination. These studies will provide a solid base for a more comprehensive research plan to be submitted as grant application to the NIH.

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Expected Research Outcomes and Benefits

The direct and immediate outcome of this project will be to demonstrate the feasibility of using dielectric barrier discharge (DBD) as a novel and non-invasive method for increasing the

efficacy of DNA-based vaccinations. Using these proof-of-concept results, we will solicit support from the NIH for more extensive developmental studies, which will advance this unique approach into human trials and, ultimately, clinical use.

The benefit of this project will be to increase the effectiveness of vaccination, which is an essential cornerstone of human healthcare. The immediate focus of this project will be the development of an approach that augments the delivery and efficacy of DNA-based vaccines that are being investigated for future use. However, because NTP-associated mechanisms of action that increase the effectiveness of DNA vaccination should similarly affect the delivery and efficacy of traditional vaccines (e.g., inactivated, attenuated, toxoid, and subunit vaccines), this project will potentially lead to approaches that can be applied to the delivery of all vaccines worldwide.

Summary of Research Completed

Specific Aim 1 Progress:
During the first half of this project, a focus was placed on the *in vitro* experimentation outlined in Aim 1. As indicated in the proposal, the goal of planned *in vitro* experiments is to explore and establish parameters in our *in vitro* model system that will be used to guide the design of *in vivo* experiments outlined in Aim 2. Variables to be investigated include DBD plasma exposure time and source waveform (frequency). Outcomes to be measured include: epithelial cell viability (including both necrosis and apoptosis); epithelial barrier function [including trans-epithelial electrical resistance (TEER) and FITC-dextran permeability; DBD plasma-induced inflammation (measured by basolateral cytokine and chemokine release); immune cell chemotaxis; and DNA uptake in DBD plasma-exposed cells.

In previous experiments performed by Dr. Miller in the A.J. Drexel Plasma Institute and in preliminary experiments performed by Dr. Krebs, DBD plasma was delivered to mammalian cells in submerged culture using an electrode designed to generate DBD plasma over a wide area of cells. These electrodes, however, were not suitable for our transwell-based polarized epithelial model system, since the electrodes were too large to be accommodated in the 24-well transwell inserts used to culture the cells. As a result, these experiments required the fabrication of a new electrode (Figs. 1A and B) with a smaller outside diameter.

The new electrode, while suitable for use in the 24-well transwell inserts, has introduced two unexpected challenges that have added additional variables to our *in vitro* experiments. The first is electrode positioning. While the electrode can be precisely positioned vertically (Fig. 1C), the electrode cannot always be positioned consistently in the center of the transwell insert, since the outside diameter of the electrode is somewhat smaller than the inside diameter of the transwell insert. If the electrode is off-center, DBD plasma may be delivered asymmetrically, causing uneven dosing or hot spots of plasma-induced cell necrosis or apoptosis. We are seeking a simple but effective engineering solution to this potential problem.

The second challenge concerns the geometry of the electrode end. The larger electrode used in large scale experiments was cylindrical with a flat end of dielectric material where the plasma

was generated. The smaller probe fabricated for use in transwell inserts has a rounded end (Fig. 1A). We are considering the effect that probe shape has on DBD plasma generation and delivery to the target cells. This aspect of our investigations will potentially affect not only the *in vitro* experiments, but also the design of the probe used for the animal model experiments (Aim 2) and the eventual translation of this concept into clinical practice.

We have moved forward with *in vitro* exposure experiments using an equipment configuration (Fig. 1D) that permits control over parameters that have been shown to modulate cell responses to DBD plasma, including exposure duration and voltage source frequency. A function generator, which was purchased for these studies, facilities experiments using DBD plasma generated with a nanosecond scale waveform, which has been shown in previous preliminary experiments to be less cytotoxic relative to plasma generated with a microsecond scale waveform (data not shown).

Experiments in Aim 1 rely on the use of a transwell-based culture system to model polarized skin epithelial cells. In this model system, cells of the human HaCaT keratinocyte cell line are grown in a transwell cell culture insert, which facilitates growth of a polarized epithelial layer and allows discrete access to the apical and basolateral sides of the cell layer. In previous (unrelated) experiments using epithelial cells of cervicovaginal origin, cells were cultured with media in the basolateral (lower) chamber and media (or a vaginal fluid simulant) in the apical (upper) chamber. Because the purpose of the current transwell system is to model the dry surface of the epidermis, however, the HaCaT cells were cultured with no media in the upper chamber. Initial cell culture efforts established a procedure that resulted in consistent cell plating with little or no losses in cell viability once the apical medium was removed and the cells were maintained at the air-liquid interface (data not shown).

The overall goal of Aim 1 is to establish DBD exposure parameters that (i) will guide the design of experiments in Aim 2 and (ii) are consistent with the use of DBD plasma as an adjuvant during DNA vaccine delivery. Since preliminary experiments have shown that exposure time and waveform frequency are determinants of *in vitro* cytotoxicity attributed to DBD plasma exposure, experiments were conducted to first explore the effect of frequency on HaCaT cells in the model system. Measured endpoints were cell viability and cell layer permeability, as assessed by MTT assay and FITC-dextran permeability, respectively.

Results of MTT assays performed after DBD plasma exposure demonstrated that HaCaT cell viability changes with frequency (Fig. 2). In these experiments, source waveform frequency was varied between 1 Hz and 75 Hz while holding exposure duration (10 sec) and electrode distance (1 mm) constant. Frequencies at or below 5 Hz reduced cell viability below 50%, indicating that DBD plasma generated at these frequencies was considerably cytotoxic. At 6 Hz and above, DBD plasma was much less cytotoxic, reducing cell viability after exposure to only 70-80% of control levels. The exceptions were exposures at 9, 10, and 15 Hz, which reduced cell viability to 50-70% relative to mock-exposed cells. These results suggest that higher frequencies, particularly frequencies at and above 20 Hz, are less detrimental to cell viability and will be suitable for subsequent *in vitro* experiments outlined in Aim 1.

The frequency-dependent effect of DBD plasma on HaCaT cells was corroborated in experiments that used cell layer permeability as an endpoint (Fig. 3). In a transwell epithelial model system, cell layer permeability to a fluorescently tagged molecule can be used to assess the barrier function of the cell layer and as an indirect measure of cell viability after exposure to a potential insult. In this experiment, cell permeability should be inversely correlated with cell viability; as viability is reduced, permeability is expected to increase. This was indeed the result obtained in experiments with HaCaT cells. At frequencies at or below 5 Hz, cell layer permeability was higher relative to the lower cell layer permeability at frequencies at and above 20 Hz.

These experiments suggest that frequencies between 20 Hz and 75 Hz will be suitable starting points for subsequent experiments involving changes in exposure duration and electrode distance. Our goal will be to define a set of exposure parameters that will be used for subsequent assessments of epithelium-induced inflammation, immune cell recruitment, and DNA reporter uptake following DBD plasma exposure. These parameters will also provide a starting point for designing *in vivo* experiments outlined in Aim 2.

Specific Aim 2 Progress:
The execution of the experimentation described in Aim 2 is predicated on results obtained from the *in vitro* experiments outlined in Aim 1. Investigations outlined in Aim 2 will be pursued in the second half of the funding period.

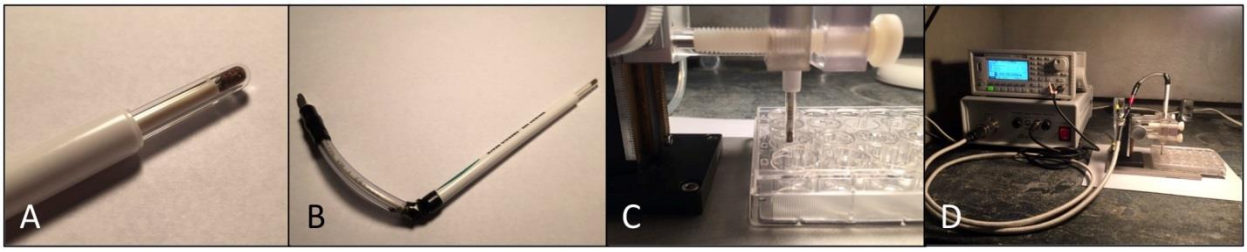

Fig. 1. DBD electrode and equipment assembled for use in the polarized skin model system. (**A**) Close-up view of the DBD electrode developed for use in the transwell cell culture system. (**B**) Normal view of the DBD electrode developed for use in the transwell cell culture system. (**C**) DBD electrode placed in a transwell insert using a Z-positioner. (**D**) Equipment used to expose cells to DBD plasma, including the high-voltage source (bottom left), function generator (top left), the electrode in the Z-positioner (center), and a receiver plate with transwell inserts (right).

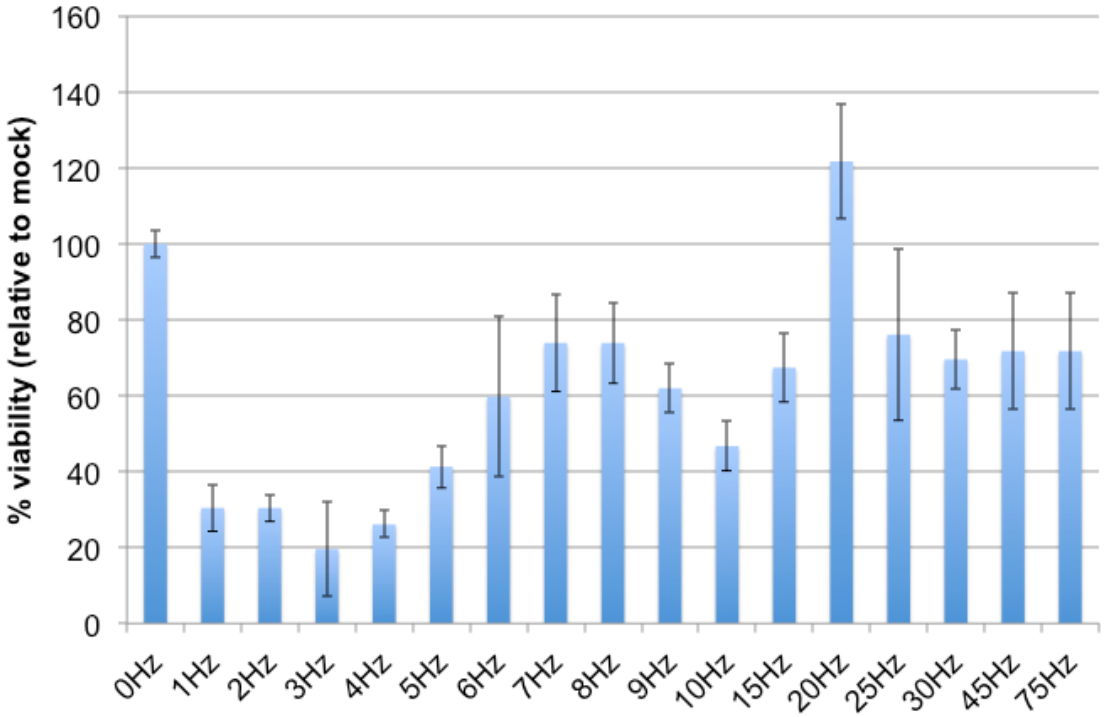

Fig. 2. Cell viability after DBD plasma exposure changes with source frequency. HaCaT cells were cultured to confluence in transwell inserts at an air-liquid interface. Cells were exposed to DBD plasma for 10 seconds at an electrode distance of 1 mm. The frequency of the voltage source was varied from 1 Hz through 75 Hz. Cell viability was assessed immediately after DBD plasma exposure using an MTT assay. The viability of cells exposed to DBD plasma is expressed as percent viability relative to mock-exposed cells (0 Hz).

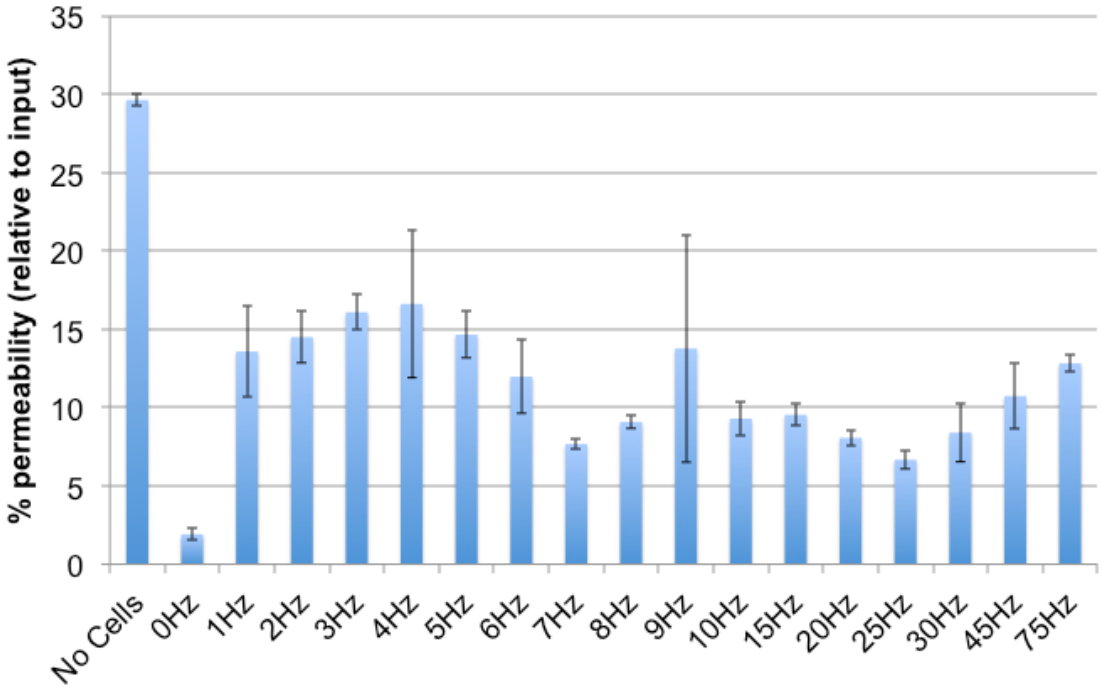

Fig. 3. Cell layer permeability after DBD plasma exposure also changes with source frequency. HaCaT cells were cultured to confluence in transwell inserts at an air-liquid interface. Cells were exposed to DBD plasma for 10 seconds at an electrode distance of 1 mm. The frequency of the voltage source was varied from 1 through 75 Hz. Cell layer permeability was measured immediately after DBD plasma exposure by adding FITC-dextran to the upper chamber and assaying fluorescence in media samples from the lower chamber 30 min after application. Permeability of cell layers exposed to DBD plasma is expressed as percent fluorescence relative to the input fluorescence. Permeability of cells mock-exposed to DBD plasma (0 Hz) was approximately 2%.

Research Project 5: Project Title and Purpose

Impaired Immune Priming and Diminished B cell Repertoires in Aging Models of Clostridium difficile Infection and Vaccination – There is a significant correlation between advanced age and risk of severe and recurrent Clostridium difficile infection (CDI). Aged individuals have a reduced ability to produce high affinity anti-toxin antibodies, but it has yet to be determined whether the defect in the elderly is due to lack of host immune priming to CDI and/or a decrease in B cell repertoire diversity making individuals more susceptible to severe disease. The purpose of this project is to identify age related functional changes in the B cell populations in the context of Clostridium difficile infection using a novel aging mouse model of CDI developed in our laboratory.

Anticipated Duration of Project

1/1/2015 – 6/30/16

Project Overview

We have established an aging mouse model of *Clostridium difficile* infection in which aged mice (>18 months old) have increased morbidity and mortality to orogastric infection with *Clostridium difficile* UK1 sublethal strains when compared to young mice. The purpose of this project is to identify age related functional changes in the B cell populations in the context of *Clostridium difficile* infection using a novel aging mouse model of CDI developed in our laboratory.

Specific Aim 1: We will use the aging mouse model of antibiotic pretreatment and hypervirulent *Clostridium difficile* orogastric spore challenge, and at time points following challenge, we will sacrifice and measure (A) toxin-specific serum and fecal IgG and IgA binding and neutralizing antibodies; (B) isolate B cells from the spleen, draining lymph node (DLN) and mesenteric lymph nodes and examine germinal center B cell surface markers and expression of co-stimulus activation markers and inflammatory cytokines; (C) measure toxin-specific B cell proliferation; and (D) measure frequency and function of toxin-specific memory B cells.

Specific Aim 2: We will examine the diversity of B cell receptor repertoire following *Clostridium difficile* infection in aged mice when compared to young adult controls. B cell repertoire is at the heart of how the immune system can adapt to nearly any pathogen it encounters and its efficacy of action degrades with age. In general following infection, the B cell repertoire undergoes affinity maturation, and expands through a cycle of hyper-mutation, proliferation and death. High affinity mutants produce memory B cells and Ig secreting plasma cells that protect the body in the case of a recurring secondary infection. In experiments outlined in Aim 2, we will create a well-calibrated experimental pipeline that will enable us to identify and sequence rare and abundant B cell clones specific to the toxins A and B of *Clostridium difficile* and compare the repertoires between aged and young adult control mice. We will harvest the spleen and draining lymph nodes (including the mesenteric LN (MLN)) from uninfected controls and infected aged and young control mice, at 10 days post infection as day

10 well-defined germinal centers in MLN are first observed post CDI. From these experiments, we will test our hypothesis that *Clostridium difficile*-specific B cells display lower levels of functional antibodies, expansion and differentiation and have limited diversity of B cell Ig repertoires in aged mice when compared to young adult controls.

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Expected Research Outcomes and Benefits

In general, aged individuals have decreased germinal center formation and a reduced ability to produce high affinity antibodies, but further understanding and identification of mechanisms underlying the defects in the elderly B cell response during initial infection and vaccination has yet to be determined in the context of *Clostridium difficile* infection (CDI). Our research group hypothesizes based on our preliminary data in a novel aging mouse model of CDI, that there is a defect in host immune priming at mucosal lymphoid sites, and that this will lead to a decrease in the diversity of the B cell repertoire. The direct and immediate outcome of this project will be to determine, using an animal model of aging and CDI, that although the aged are able to make low affinity, short-lived antibody, the capacity of their serum to neutralize C. difficile toxins is substantially decreased and the diversity of the antibody epitopes is diminished. At the conclusion of these studies we will be able to better design molecular vaccine adjuvants that bypass and/or boost these defective immune priming pathways in the elderly resulting in enhanced protective antibody in our DNA vaccine platform in the elderly. By 2050, it is predicted that almost 40% of the population in the United States will be >60 years of age, meaning that a previously unparalleled number of individuals will be at risk for CDI.

Summary of Research Completed

Specific Aim 1 Progress:
We have spent the first part of the budget year optimizing the methodologies and *Clostridium difficile* orogastric spore doses for use in the aged mouse experiments in the second half of the budget year. We carried out experiments to gather preliminary data utilizing an aging mouse model of antibiotic pretreatment and hypervirulent Clostridium difficile intragastric spore challenge **(Figure 1 panel A)**, and observed decreased survival of aged mice (>18 months) to lower, doses of spores when compared to young adult mice (4-6 months). Mice were exposed to

a mixture of antibiotics containing kanamycin (0.4mg/ml), gentamicin (0.035mg/ml), colistin (850 U/ml), metronidazole (0.215mg/ml), and vancomycin (0.045mg/ml) that has been used previously to disrupt the intestinal microflora in small animal models. Following this treatment, an injection with a single dose of clindamycin (10 mg/kg) was given, followed by C. difficile challenge the next day at doses C. difficile UK1 -10^4 or 10^5 cfu (n = 5), respectively, for each group plus a naïve, non-infected group of were used. Animals were observed daily for the duration of the experiment for mortality and morbidity and the presence of diarrhea, weight loss, loss of appetite, lethargy/dehydration. As shown in **Figure 1 panel B,** we observed that young mouse controls survived challenge doses of 10(4) or 10(5) CFU of UK1 spores, while mice >18 months of age did not survive challenge with 10(5) CFU, and only 40% of aged animals survived 10(4) CFU challenge dose. These data provide the basis for examining whether underlying aged associated defects in host immune responses to the UK1 bacterial challenge and toxin secretion would render aged models more susceptible to reinfection and recurrent infection if the germinal center B cell response is not established to lead to memory B cell formation in the intestine, where the bacteria germinates and causes disease. Therefore, we set out to establish methodologies to detect the quantity and quality of the toxin-specific B cells in response to infection with *Clostridium difficile*. **Our hypothesis** is that in an aging cohort, severe CDAD and recurrent CDI are associated with lower levels anti-toxin serum IgG, lower B cell diversity and repertoires of co-stimulatory molecules and toxin-specific pro-inflammatory cytokines, defective priming of naïve B cells to initial toxin exposure, a larger frequency of "exhausted" memory B cells and presence of less diverse B cell repertoire post-infection. 3 days following infection with Clostridium difficile (Figure 1 panel A), we isolated splenocytes and mesenteric lymph nodes (MLNs) and carried out flow cytometry using the following surface markers on follicular T cells (Tfh) and germinal center B cells (GCb): CD3, CD4, TCRbeta, CXCR5, and PD1 (Tfh) and IgD, CD19, CD38 and GL7 (GCb). As shown in **Figure 2 panel A,** we did not observe differences in the frequency of MLNs or the absolute # of MLN GCb cells. Moreover, **(Figure 2 panel B)** we did **not** observe any changes in the frequency (%) or absolute # of Tfh cells in the **spleen** when we compare young uninfected controls (YC), old uninfected controls (OC), or young controls that were infected with UK1 (YCDI) and aged mice infected with UK1 (OCDI). However, as shown in **Figure 2 panel C,** we **did** observe **increased frequency** and absolute #'s of MLN Tfh, suggesting a mucosal-specific defect in aging mice. When we presented these data to the research team of Dr. Uri Hershberg and Dr. Mike Cancro, it was suggested that we are looking too early post infected to identify GC and Tfh responses as most germinal center reactions occur 14-20 days post infection or vaccination. In addition, being able to identify antigen specific B cells using a C. diff toxin-conjugated fluorochrome to our panel would improve the recognition of antigen specific B cells post infection, not just the total frequency of these cells. Therefore, with the help of the Cancro lab, we developed a new improved flow panel to detect Tfh cells and GCB cells shown in **Table 1.** As shown under **Table 1,** we have completed titrations and compensation testing of these 2 flow cytometric panels. An accomplishment to date is the development of a more complete phenotyping panel of antibodies that include previous markers used in experiments in Figure 2, with the addition of (IgD, IgM, B220, PNA, CD138, CD19, Fas, and a dump channel) for B cells, as well as the addition of the antigenic marker, toxoid A and B. Taken together, we now have the following assays in place and tested for the upcoming aging infection and vaccination study: (A) serum and fecal IgG and IgA binding using toxoid-coated ELISA plates and qualitative neutralizing antibody

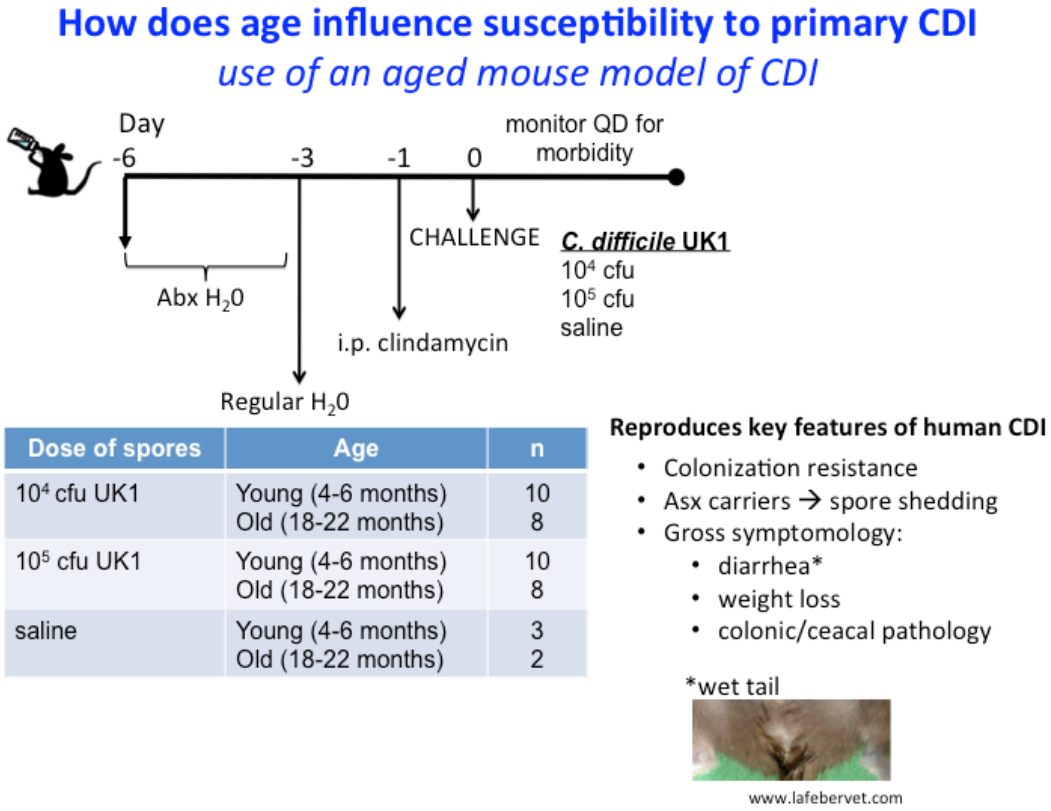
measurement in a cell rounding neutralization assay; (B) ability to isolate B cells from the spleen, DLN and mesenteric lymph nodes and examine germinal center B cell surface markers and expression of co-stimulus markers using flow cytometry (C) frequency and function of toxin-specific memory B cells using B cell ELISPOT assay using bone marrow derived memory cells. As shown in **Figure 3,** we are able to detect toxin-specific IgG and IgA in sera and fecal specimens from young and old mice post vaccination. An ELISA was used to determine levels of antigen-specific IgG in mouse serum and stool. Mouse blood samples were harvested by submandibular bleed, and subsequently, sera or fecal supernatants were analyzed individually within each experimental group, or pooled, respectively. Ninety-six-well EIA plates were coated for 2 hours at room temperature or overnight at 4°C with either 0.5 μg/ml of coating antigen (recombinant TcdA RBD or TcdB RBD) or a serial dilution of purified mouse IgG (Sigma) for creation of a standard curve. Plates were washed and blocked against nonspecific binding with 3% bovine serum albumin for at least 2 hours at room temperature. Sera or fecal sups from immunized mice were diluted in blocking buffer, added to wells in duplicate, and incubated at room temperature for 2 hours or overnight at 4°C. Bound antibodies were detected with horseradish peroxidase–labeled goat antimouse IgG or IgA, and developed with substrate 3,3′,5,5′-tetramethylbenzidine (TMB) H_2O_2 .The color reaction was stopped with 2N H_2SO_4, and the absorbance at 450 nm was read using an EL312 Bio-Kinetics microplate reader and standard curves were created by first plotting the OD_{450} values against the corresponding concentration of the purified mouse IgG. A linear regression analysis of the linear part of the curve provided a line of best fit as well as an equation for this line. For quantitation of αRBD IgG in mouse sera, the "x" variable within the equation was substituted for an OD_{450} value taken from the linear part of the dilution curve. Finally, the equation could be solved for the "y" variable, or concentration (ng/μL) of IgG within the sample. These antigen specific measurements of antibody post challenge or vaccination will be utilized moving forward with the addition of IgM measurement since this is the first Ig that is expressed following infection of vaccination.

Specific Aim 1 Progress:

The goal of aim 2 is to acquire the relevant toxin-specific B cell repertoire(s) by using FACS analysis and sorting, PCR amplification and high throughput sequencing. Using a FACS Aria sorter we will use surface markers as described earlier in our progress report to isolate the mature naïve and germinal center B cells from spleen, germinal center B cells from MLN and plasma cells from MLN. The first stage of Aim 2 pipeline includes FACS single cell sorting of relevant B cell subsets from the spleen and MLN. To date, we have optimized the isolation and detection of antigen-specific B cells in the spleen and MLN post infection, and the next step is to show that these cells can be sorted using FACS aria.

Figure 1. Advanced age increases susceptibility to primary Clostridium difficile infection using a an aging mouse model of antibiotic pretreatment and hypervirulent Clostridium difficile intragastric spore challenge.

A.



B.

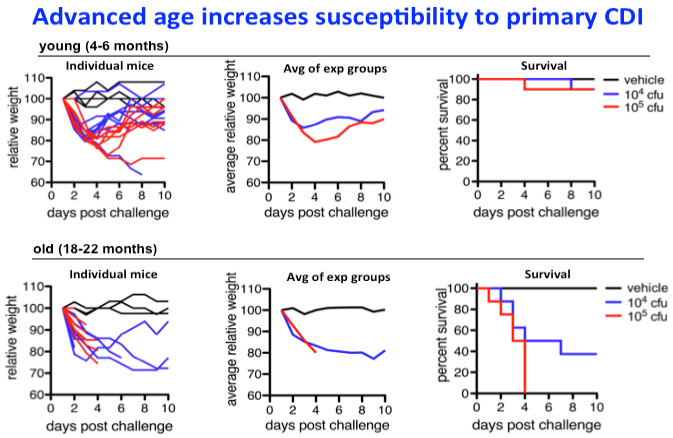
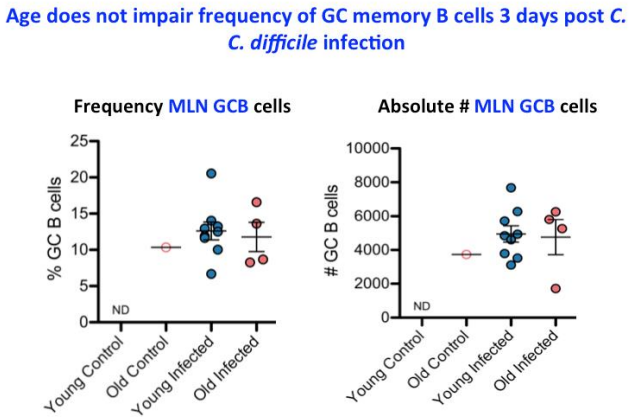


Figure 2. Advanced age does not impair the frequency of MLN GC memory B cells or splenic Tfh cells 3 days post Clostridium difficile infection using a an aging mouse model of antibiotic pretreatment and intragastric spore challenge. Frequency and Absolute # of mucosal T follicular helper cells in AGED mice is altered 3 days post Clostridium difficile infection. Legend: young uninfected controls (YC), old uninfected controls (OC), young controls that were infected with UK1 (YCDI) and aged mice infected with UK1 (OCDI).

A.



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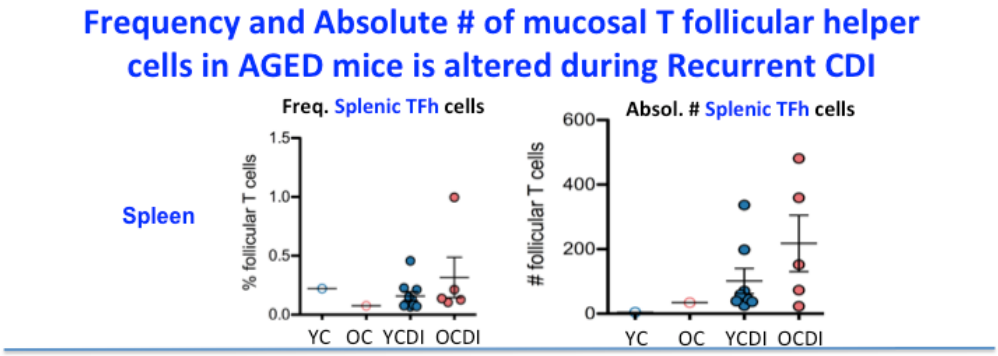

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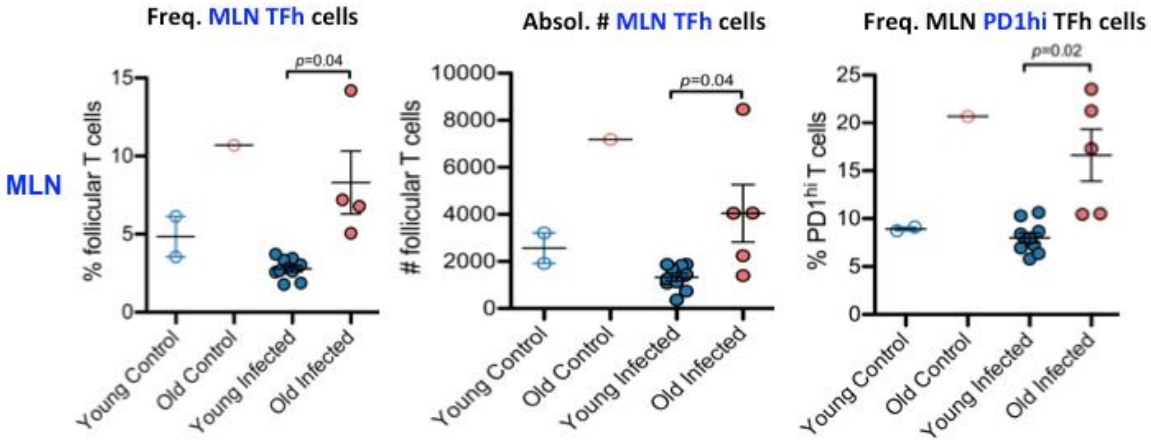


Table I. Flow cytometry antibody panel to detect antigen specific (toxin) B cells in splenic and draining lymph node germinal centers and Tfh cells at these sites.

B cell Panel	Fluorophore	Antibody
	APC	IgD
	PE	CXCR5
	PE-Tx Red	IgM
	PerCP-Cy5.5	B220
	FITC	PNA
	BV605	CD138
	BV785	CD19
	PE-Cy7	Fas
DUMP	PE-Cy5	F4/80
	PE-Cy5	GR1
	PE-Cy5	CD4
	PE-Cy5	CD8
	PE-Cy5.5	Toxoid A and B
Tfh cell panel	BV785	CD19
	PE	CXCR5
	Pe-Cy5	CD4
	PE-Cy7	PD-1
	BV421	TCRBeta
	APC	CD62L
	AF700	CD38

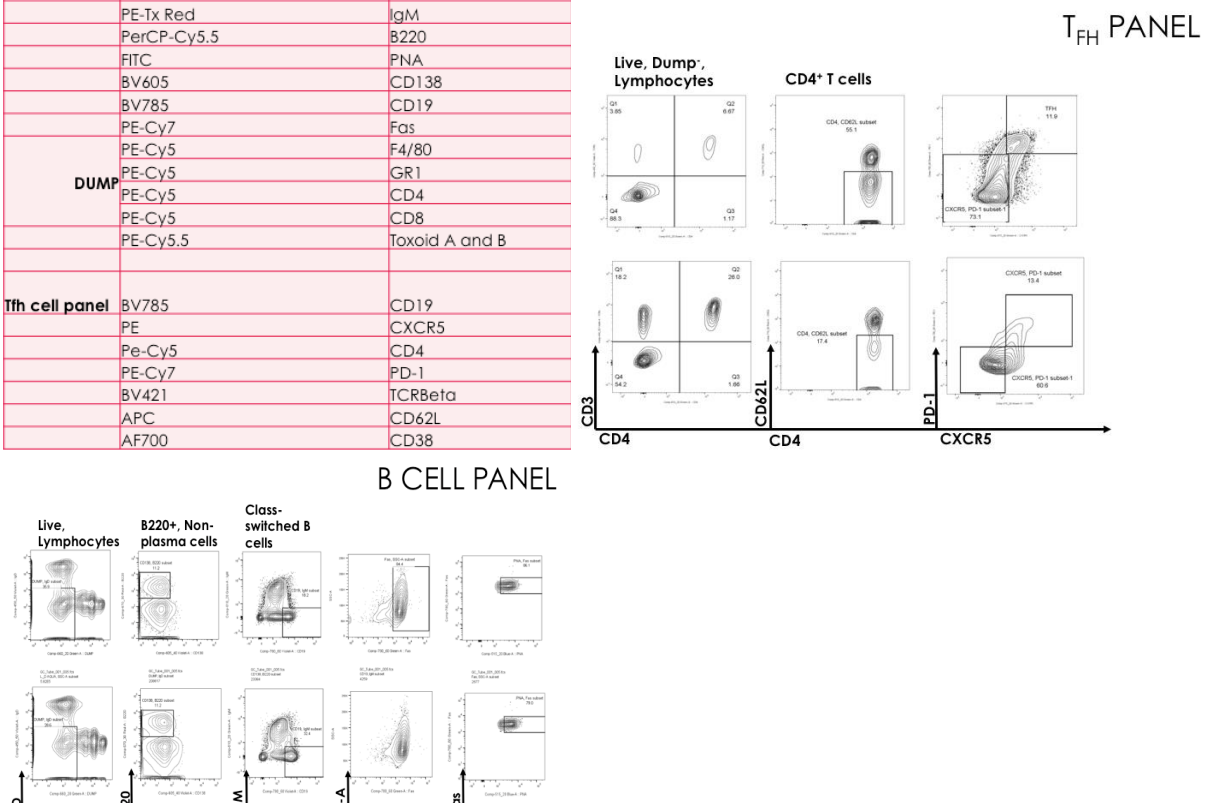
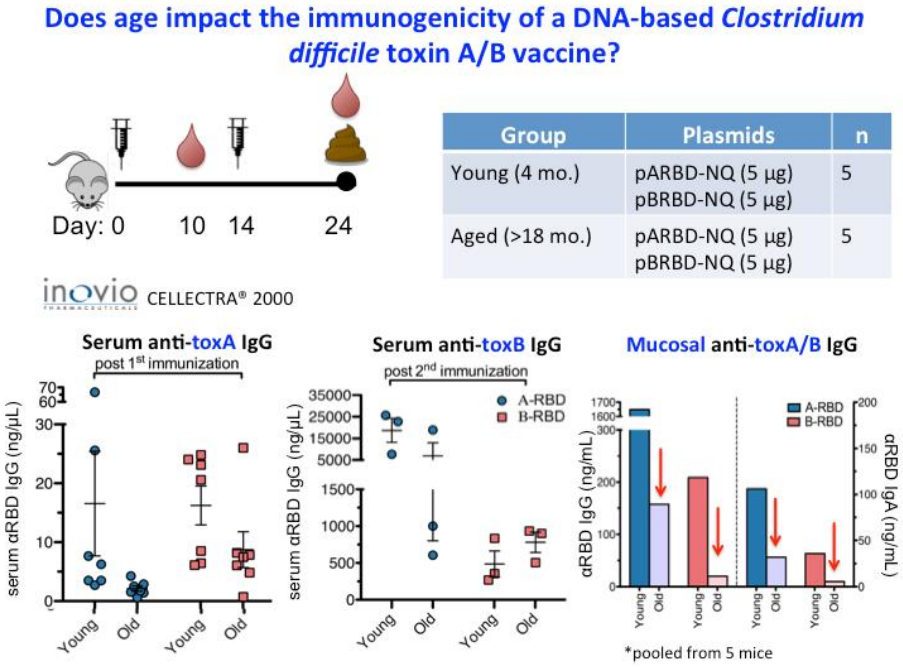


Figure 3. On Day 12 post-first immunization with toxin based DNA vaccine, the younger mice had significantly higher anti-ARBD IgG titers (25ug/ul) than older mice (5ug/ul). Although 10 days post-second immunization both groups had comparable anti-ARBD IgG titers (~500 ug/ul IgG), anti-toxin B-RBD IgG responses in older mice remained lower throughout the experiment.



Research Project 6: Project Title and Purpose

Neuroprotective Effects of Inhaled Carbon Monoxide in a Piglet Model of Hypoxia – In a newborn piglet model of hypoxic cerebral injury, increased activation of the enzyme Ca++/calmodulin kinase (CaM Kinase) IV localized in the nucleus is seen within one hour of exposure to hypoxia. The focus of this project is to build on these observations and test whether carbon monoxide (CO) inhalation given therapeutically at low safe doses after hypoxic cerebral injury improves functional disability scores, attenuates brain pathology, and/or inhibits caspase-9 /caspase-3 activation in a neonatal piglet model of hypoxic cerebral injury. We envision CO being present in neonatal intensive care units, akin to inhaled nitric oxide, to be administered at the bedside via nasal cannulas or ventilator if needed.

Anticipated Duration of Project

1/1/2015 – 12/31/2016

Project Overview

BROAD RESEARCH OBJECTIVES: Given that we expect that CO will have benefits, we envision CO being present in neonatal intensive care units, akin to inhaled nitric oxide, to be administered at the bedside via nasal cannulas or via a ventilator if needed. SPECIFIC RESEARCH AIMS: 1. Evaluate functional disability; 2. Evaluate gross histology, neurodegeneration, traumatic axonal injury, reactive astrocytosis, and breakdown of the blood brain barrier; 3. Evaluate caspase-9 and caspase-3 activity. RESEARCH DESIGN: The animals will be ventilated under either normoxic or hypoxic condition. Hypoxia will be induced by lowering the FiO_2 to 0.06 for 60 minutes. Minute ventilation will then be adjusted to maintain an arterial partial pressure of carbon dioxide of 35-45 mmHg and the FiO_2 in the hypoxic groups will be increased to 0.21. A calibrated CO gas mixture of 250 ppm will be administered. All piglets will be weaned from the ventilator and extubated after the 60 minutes of normoxia/hypoxia. Experiment 1A: *Evaluation of Functional Disability*: An examination by an observer blinded to group assignment will be performed 24 hours, 3 days, and or 7 days after completion of normoxia/hypoxia to determine functional disability. The animals will be ranked 0 - no disability, 1 – mild disability, 2 – moderate disability, 3 – severe disability, or 4 - death. The open field, T-mase, and inclined beam studies will be performed. Experiment 1B: *Evaluation of Gross Histology, Neurodegeneration, Traumatic Axonal Injury, Reactive Astrocytosis, and Breakdown of the Blood Brain Barrier*: At 24 hours, 8 animals in each group will be anesthetized and euthanized by exsanguination. The remaining animals will be sacrificed at 7 days. The brain will be post-fixed and stained for assessment of gross histology, neurodegeneration, traumatic axonal injury, and reactive astrocytosis. Experiment 1C: *Evaluation of Caspase-9 and Caspase-3 Activity*: At 24 hours or 7 days, the brain will be analyzed for caspase-9 activity and caspase-3 activity.

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Expected Research Outcomes and Benefits

Carbon monoxide (CO), like nitric oxide before it, may prove to be a therapeutic option and a new and novel approach to neonatal encephalopathy and other neuropathologies. This preclinical study is designed to study the effectiveness of inhaled CO administered in a piglet model of neonatal encephalopathy to improve functional disability scores and reduce brain pathology. Safety and tolerability studies in a controlled Phase I FDA approved safety study showed no adverse events in healthy volunteers up to 12% COHb which is our target dose in our proposed studies herein. Thirty-one patients have already received CO as an inhalational therapy intraoperatively during kidney transplantation in a Phase II clinical trial. An inhaled-CO-delivery device for clinical use that meters gaseous CO in mg/kg units to more accurately dose patients based on body weight has been developed by Ikaria and is available with integrated safety features. The clinical role of CO as a neuroprotective and neurotherapeutic agent has been suggested. Few studies have evaluated CO as a therapeutic modality for brain injury. One study showed that inhalation of 125-250 ppm CO immediately at the onset of reperfusion in a mouse model of focal brain ischemia resulted in reduction of total hemispheric infarct volume. In our laboratory, inhalation of CO (250 ppm) before cardiopulmonary bypass and deep hypothermic circulatory arrest in a neonatal piglet model resulted in less apoptosis in the brain and a shift in the lactate/glucose index. Preclinical studies of protective conditioning show less pathology in models of epilepsy, stroke, hypoxia-ischemia, traumatic brain injury, and craniocerebral tumor resection. Results from this study will allow us to move toward clinical studies in the use of inhaled CO in the patient with neonatal encephalopathy.

Summary of Research Completed

Specific Aim 1 Progress:
We have completed hypoxia studies on two piglets. Neurobehavioral studies have been completed and the brains have been harvested. Brains of piglets that have undergone the protocol are being prepared for gross histology, neurodegeneration, traumatic axonal injury, reactive astrocytosis, and breakdown of the blood brain barrier. Brains of the piglets that have undergone the protocol are being prepared to evaluate caspase-9 and caspase-3 activity.

Hemodynamic monitoring during the experiment demonstrated a drop in systolic pressure, diastolic pressure, and mean arterial blood pressure during the hypoxic insult. Saturations decreased to 0. Blood pressure and blood gas changes returned to normal during the resuscitative phase. Temperature was maintained during the entire procedure in the piglets. (Table 1) Blood gases drawn during hypoxia showed a drop in p02, bicarbonate, base, and saturation. (Table 2) (Figures 1 and 2)

One of the piglets had major neurologic findings by 24 hours. These included extensor posturing and inability to right himself. This animal was sacrificed at 24 hours and the brain harvested for the above studies.

Functional disability scoring (Table 3), open field (Figure 3), T-maze (Figure 4), and inclined beam studies (Figure 5) were completed on the piglet that survived to Day 8. Subsequently, the animal was sacrificed and the brain harvested for the above studies.

Specific Aim 2 Progress:
Brains of two piglets that have undergone the protocol are being prepared for gross histology, neurodegeneration, traumatic axonal injury, reactive astrocytosis, and breakdown of the blood brain barrier.

Specific Aim 3 Progress:
Brains of the piglets that have undergone the protocol are being prepared to evaluate caspase-9 and caspase-3 activity.

Table 1 – Hemodynamic Studies

PIGLET	LOWEST TEMPERATURE	LOWEST HEART RATE (DURING HYPOXIA)	LOWEST SATURATION (DURING HYPOXIA)	STARTING MAP (BEFORE HYPOXIA)	LOWEST MAP (DURING HYPOXIA	END MAP
1	38.1	153	0	74	32	62
2	37	61	0	62	26	82

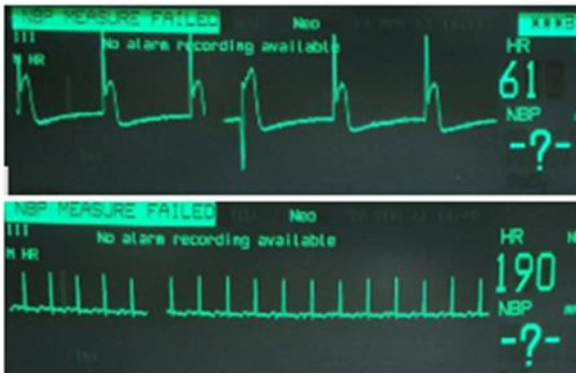
Table 2 – Lowest HCO_3 and Base during Hypoxia

PIGLET	LOWEST HCO_3	LOWEST BASE
1	7.8	-24.6
2	11.2	-13.9

Table 3 – Functional Disability Scoring

	DESCRIPTION
0	NO DISABILITY
	(able to run, explore the environment, and feed from the trough)
1	MILD DISABILITY
	(gait disturbances but able to ambulate, explore the environment, and feed from the trough)
2	MODERATE DISABILITY
	(unable to walk but alert and able to crawl, feeds with assistance)
3	SEVERE DISABILITY
	(unable to crawl, not able to feed even with assistance)
4	DEATH

Figure 1 – Piglet intubated with lines in place



During hypoxia

After resuscitation

Figure 2 – EKG of piglet during hypoxia and after resuscitation

Figure 3 – Open Field Study

Figure 4 – T-Maze Study

Figure 5 – Inclined Beam Study

Research Project 7: Project Title and Purpose

Non-invasive Monitoring of Cerebral Edema in Real-Time Using a Novel Near Infrared Spectroscopy Monitoring System. – The development of cerebral edema following a hypoxic ischemic (HI) event in newborn infants carries high risk for brain damage and death. Currently, edema is detected by CT/MRI scan. However, these infants are often critically ill and cannot tolerate transport to undergo imaging. We developed an alternative portable and non-invasive NIRS-based neuroimaging device to monitor changes in the blood and water content of the brain. We will perform pre-clinical studies in piglets to demonstrate the ability of the device to detect the evolution of edema in real time in a well-characterized animal model of HI and neuroprotection. This novel cerebral edema monitoring system can then be used to assess patient status in a safe, accurate and timely manner.

Anticipated Duration of Project

1/1/2015 – 6/30/2016

Project Overview

Aims: Hypothermia has emerged as the standard of care for treatment of neonatal hypoxic-ischemic encephalopathy (HIE). Our goal is to perform pre-clinical studies in newborn piglets to demonstrate the ability of our novel and portable NIRS-based neuroimaging device to detect the evolution of cerebral edema in real time in a well-characterized animal model of neonatal hypoxia-ischemia (HI). We will also test the hypothesis that cerebral edema contributes to brain injury after HI, and that management of cerebral edema using hypothermia as a neuroprotective intervention will decrease subsequent brain injury. The work to be performed is as follows:

Specific Aim 1: We will perform studies using our novel cerebral edema monitoring system in both normoxic piglets and piglets exposed to a hypoxic ischemic insult to demonstrate the ability of this method to penetrate through the cranium into the brain proper of newborn piglets and detect changes in cerebral oxygen and water content that occur during and after cerebral HI.

Specific Aim 2: We will correlate changes in NIRS-derived water signal with anticipated changes in intracranial pressure and brain water content and water channel protein expression at 4 hours and 24 hours after HI in both normothermic (NT) and hypothermic piglets, and compare these measurements to those in normoxic piglets. We will also correlate the observed changes with biochemical markers of brain injury and functional outcomes at 4 hours and 24 hour after HI.

Design and Methods: All animal experiments will be performed with 3-5 day old piglets using protocols approved by the Drexel University IACUC and randomized to one of 4 interventions: Normoxia-NT, Normoxia- Hypothermia (HT), HI-NT, and HI-HT. The piglets will be anesthetized and ventilated with normal or with low levels of oxygen for 1 hr. Changes in cerebral oxygen and water content will be monitored using the NIRS device. The piglets will then be maintained at normal temperatures or cooled to 33°C for 4 hours. Cerebral water content (ml water/gram tissue) will be determined using the ratios of wet-dry weight/wet weight of samples of the cerebral cortex measured before and after incubation for 72 hours at 90°C, and aquaporin water channel protein expression will be measured on Western blots as a marker of cerebral edema.

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Expected Research Outcomes and Benefits

The funding provided by this CURE grant will support essential preclinical testing in an animal model needed to establish the ability of a newly developed NIRS-based cerebral edema monitoring device to measure safely and accurately in real-time both oxygen and water content in the brain. This project also tests the hypothesis that reducing cerebral edema by hypothermia for neuroprotection following hypoxia-ischemia will improve markers of brain damage in a well-established model of hypoxic brain injury in newborn piglets. The multidisciplinary collaboration brings the engineering and device development side together with the potential end users of the device on the clinical side. The ability to detect cerebral edema early using a portable, non-invasive strategy provides clear advantages over traditional imaging techniques and can provide valuable information to inform medical management across a broad spectrum of neonatal, pediatric and adult patient populations. The development of cerebral edema following brain injury carries high risk for brain damage and death. Hence, sharing the insight gained from this novel and patented technology in the setting of newborn hypoxic brain injury through scientific publications and commercialization efforts can potentially enhance the state-of-art of technology used to detect brain damage at early stage of disease at time of diagnosis, and thus reduce death and neurologic disability in wide range of pediatric and adult diseases. The ultimate goal is to provide to the community at large the ability to detect cerebral edema early and accurately in situations ranging from pursuit of neuroprotective therapy to save the brain of asphyxiated infants, to first-responder triage of head trauma victims in an urban setting or in combat zone, to management of patients with head bleeds, post cardiac arrest or stroke and more.

Summary of Research Completed

The milestone for the first period of the project was to enroll 16 piglets, at least 2 in each study group, perform biochemical analysis on brain samples, and conduct interim statistical analysis of data collected so far. To date, the project has progressed well along this timeline and has achieved milestones set for the first period, as detailed below.

Subject recruitment: Seventeen piglets have been studied so far. The specific break down among the study groups is as follows: (1) Normoxia-Normothermia-4hours: 5; (2) Hypoxia-Normothermia-4 hours: 8; (3) Hypoxia-Hypothermia-4 hours: 2. Two normal piglets were also studied as a reference group providing the normal values for the parameters included in this study specifically to indicate the normal cerebral content in healthy non-stressed non-instrumented 3-5 day piglets.

Experimental protocol: the approved animal experiments outlined in the original application has been successfully followed and conducted as planned. Anesthetized piglets were exposed to either hypoxia ischemia (HI) [FiO_2 0.07 for 1 hr and hypotension (40% decrease in systolic BP)], then returned to FiO_2 0.21 to restore O_2 and BP for 4 hrs (HI-4Hr; N=7), or were maintained with normoxic (Nx) FiO_2 0.21 for 4 hr (Nx-4Hr, N=6). A custom made 3-channel sensor was placed on the head at baseline and maintained throughout the experiment, and changes in light attenuation were coupled to changes in deoxyHb, oxyHb and water. Piglets were placed on a

circulating water blanket, and those assigned for hypothermia were cooled to core body temperature of 33±0.5°C for 4 hours after HI/Nx exposure. Normothermic piglets were maintained at normal body temperatures, which in piglets is around 39°C. Cerebral water content was determined as wet-dry weight/wet weight of samples of the cerebral cortex before and after incubation for 72 hrs at 90°C. The height of the cerebrospinal fluid column was recorded, with an ICP ≥11 cm H_2O considered an elevated value based on published norms.

Specific Aim 1 Progress: We have successfully performed studies in both normoxic piglets and piglets exposed to a hypoxic ischemic insult that demonstrated the ability of our novel cerebral edema monitoring system to penetrate through the cranium into the brain proper of newborn piglets and detect changes in cerebral oxygen and water content that occur during and after cerebral HI.

(A) OxyHb/deoxyHb signal: As expected, deoxyHb signal increased and oxyHb signal decreased during hypoxia, and returned to baseline after reoxygenation. The tracings remained steady throughout the monitoring period in normoxic piglets. Arterial oxygen tension dropped significantly during the hypoxia exposure compared to normoxia (26±2 vs 91±2 mmHg; $p<0.05$ vs. Nx) Systolic blood pressure (SBP) in normoxia and baseline SBP prior to onset of hypoxia were comparable (91±5 vs 103±6 respectively). Blood pressures dropped significantly during hypoxia by ~55±7% ($p<0.05$ vs pre-HI) and partially recovered to ~75±3% after reoxygentaion. Blood pressures in normoxic piglets remained steady within 15±3% from baseline.

(B) Water signal: The tracings from the water channel increased progressively from baseline over the 4 hour period following HI and peaked at 28.50±6.02 ΔmV from baseline. In contrast, the tracing from the water channel in normoxic piglets increased by only 5.09 ΔmV from baseline over a 4 hour period ($p<0.05$ vs HI). Cerebral tissue water content measured on a piece of cerebral cortex was significantly increased in piglets exposed to HI compared to normoxic controls (6.00±0.19 vs 5.47±0.38 ml H_2O/g dry wt, $P<0.05$ vs Nx). As a reference, water content in healthy 3-5 day old piglets was 5.45±0.00 ml H_2O/g dry wt. All piglets in the HI group tested for intracranial pressure had elevated pressures ≥11cmH_2O, compared to none in the normoxic groups (12.5±1.6 vs 9.5±1.0 cm H_2O, $p<0.05$ vs Nx).

Taken together, these data demonstrate well that light from all 3 optodes on the custom made channels for oxyHb, deoxyHb and water on our device was able to penetrate through the cranium and into the brain proper of newborn piglets and reflect real change in cerebral tissue physiology that occurred during and after cerebral HI (Figure 1).

Specific Aim 2 Progress: We began the work needed to correlate the observed NIRS changes to physiologic and biochemical parameters and preliminary findings have been encouraging so far as outlined below. Long term functional and histological correlates will also be addressed as requested by the reviewers. However, this will require more experiments that are planned for the second period of the project to address these goals.

(A) Our preliminary data indicate a good correlation between changes in NIRS-derived water signal and changes in and brain water content at 4 hours in normothermic piglets. We found a significant positive correlation between the magnitude of change in water signal (ΔmV) and cerebral tissue water content (ml H_2O/g dry wt) with a Pearson correlation coefficient R of

0.851, $p<0.05$ and n=6. As expected, there was a highly significant positive correlation between ICP and cerebral tissue water content ($R=0.912$; $p<0.05$, n=5). There was a positive correlation between change in water signal and ICP ($R=0.753$), however, it did not reach statistical significance at present, and more experiments are planned. Data for ICP was available from only a subset of the animals due to technical difficulties and/or biological instability that hindered accurate collection of ICP in some of the animals. These findings will be confirmed in analysis and experiments planned for the second period of the project.

(B) Preliminary data from the 2 HI piglets that underwent cooling indicate the optodes are able to convey the signal during hypothermia as well. Water content was comparable to normal piglets (5.54), and the water signal did not increase, suggestive of neuroprotection from cerebral edema with hypothermia (Figure 2). Further analysis and comparisons to normoxic hypothermic piglets will continue in the second period of this project.

(C) Biochemical studies: Preliminary Western blot analysis of aquaporin-4 protein expression in normothermic piglets suggest an increase in aquaporin water channel in the cerebral cortex at 4 hours after HI compared to Nx (Figure 3). Further experiments are underway to confirm.

While the 3-channel probe was on the head of the piglets we noticed a dramatic increase in the waveform of the water signal immediately after a saline bolus was given to hypoxic versus normoxic piglets (figure 4). We suspected that this change from baseline in the tracing over a 2-minute period before and after rapid i.v. bolus of 10 ml/kg saline can be used to assess the status of cerebral autoregulation in sick animals in real time. Impaired ability of cerebral blood vessel to autoregulate blood flow to the brain in the face of decreasing systemic blood pressure leads to cerebral hypoperfusion, and predisposes to ischemic brain damage. We tested the hypothesis that NIRS-derived cerebral water signal detects impaired cerebral autoregulation following HI in newborn piglets. In normoxic piglets, peak changes in NIRS-derived signals (Δabsorbance) following saline boluses were 1.09 ± 0.06 for deoxyHb, 1.35 ± 0.44 for oxyHb and 1.91 ± 0.44 for water. In contrast, peak changes in NIRS- signals following HI were 2.00 ± 0.546 for deoxyHb, 2.70 ± 0.19 for oxyHb and 6.37 ± 2.60 for water. The data show larger disturbances in NIRS signal following a saline bolus in HI- compared to normoxic piglets for the three chromophores studied, indicating impaired cerebral autoregulation following HI. The magnitude of change was most pronounced when water is used as the chromophore of interest. These preliminary data demonstrate the ability of our novel NIRS monitoring system to detect impaired cerebral autoregulation following HI in piglets. The novel water channel provided greater level of resolution to detect disturbances in cerebral perfusion compared to either deoxyHb of oxyHb. We suggest that the use of bedside NIRS technology will provide valuable information about the state of the cerebral circulation in infants with HIE that can guide hemodynamic support.

Figures:

Figure 1. Detection of Cerebral Edema Using a 3-Channel NIRS in Normoxic and Hypoxic Piglets

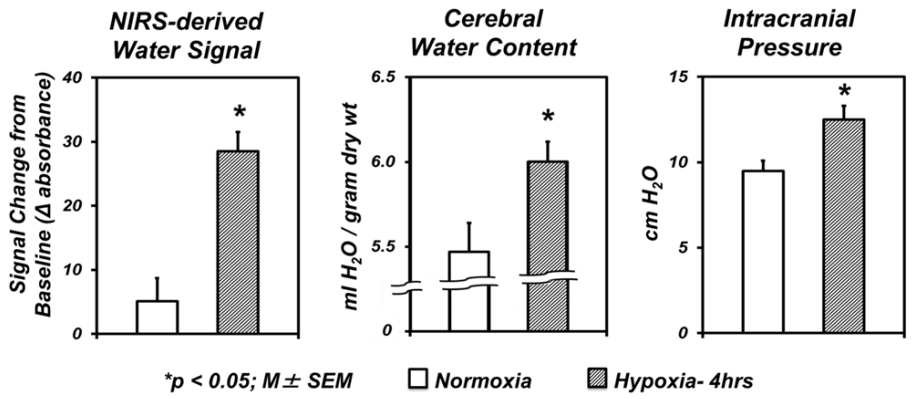


Figure 2. Representative tracings showing changes in NIRS signals following Hypoxia in Hypothermic Piglets

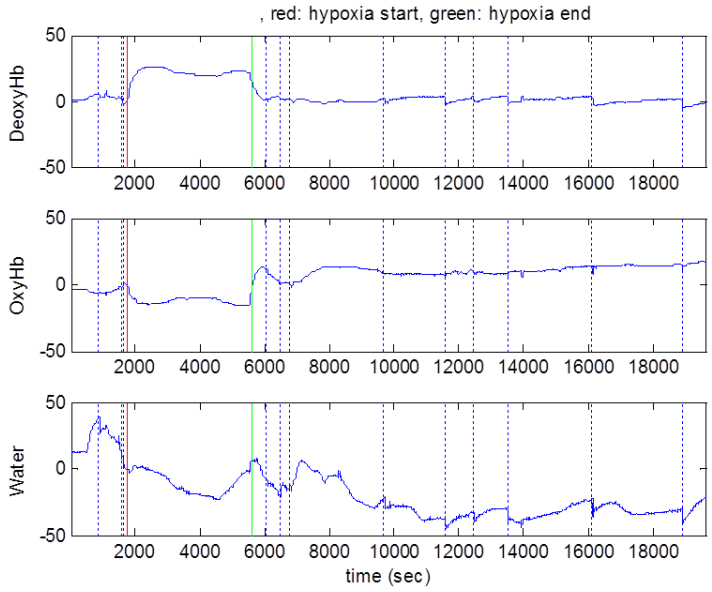


Figure 3. Western blot of Aquaporin-4 (Crude gel, needs repeat)

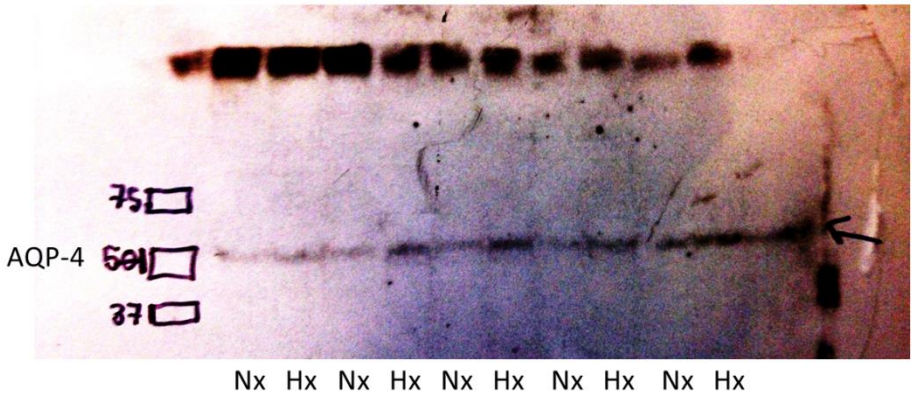
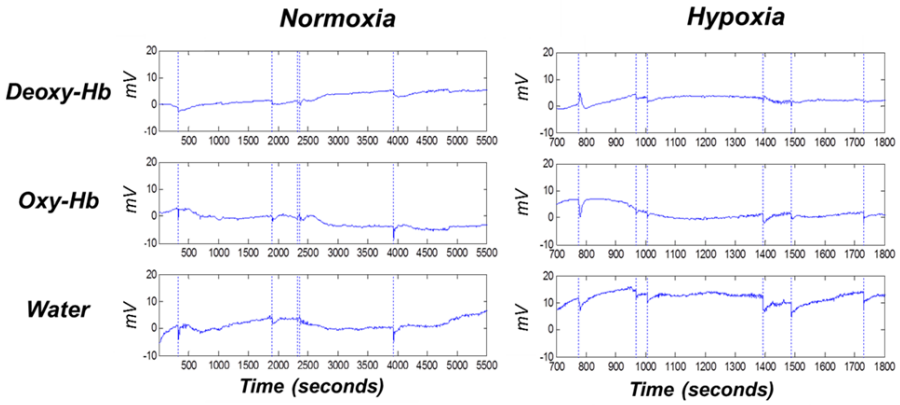


Figure 4. Representative tracings showing changes in NIRS signals following saline boluses



Research Project 8: Project Title and Purpose

Metabolic Control of Neurogenesis Via Histone Acetyltransferase Tip60 – The purpose of this research is to elucidate the metabolic control of a process called neurogenesis critical to a therapeutic intervention for a number of neurodevelopmental and neurological diseases such as autism spectrum disorder and Alzheimer's disease.

Anticipated Duration of Project

1/1/2015 – 12/31/2015

Project Overview

This research addresses the central hypothesis that metabolism is a pathophysiological mediator of neurological dysfunction. Specifically, this project will elucidate the modulation of metabolic pathways by the histone acetyltransferase Tip60 during neurogenesis. Elucidating this function will enable 1) improved design of targeted histone deacetylase inhibitors and 2) design of metabolic interventions to reduce morbidity and mortality from diseases where histone modifications are known to play a role. Specific Aim 1. Elucidate metabolic substrate utilization during neurogenesis in dTip60 wild type versus mutant dTip60E431Q. Specific Aim 2. Correct defective neurogenesis in Tip60 mutants by metabolite replacement or rescue dTip60. To accomplish these aims, cutting edge liquid chromatography- mass spectrometry methods, combined with basic molecular biology tools will be employed by a number of researchers.

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Expected Research Outcomes and Benefits

Neurodegenerative and neurodevelopmental disorders display an increasing prevalence and the impact of these diseases, including Alzheimer's disease and autism spectrum disorder, is enormous. The high number of co-morbidities with these disorders significantly impacts the quality of life for patients and family members, and this research closely relates to modalities of treatment currently in development for these disorders. A better understanding and more useful model systems for the development of the nervous system is a widely recognized need that this research pursues.

Summary of Research Completed

The research completed to date was directly in-line with the proposed timeline for Aim 1 and Aim 2. Growth optimization and analytical method development have both proceeded as per timeline despite a major unanticipated challenge in Aim 1 as outlined below. Notably, the analytical isotopic tracer methodology, contained in aim 1 and funded by this grant, contributed to a highly collaborative paper currently in peer-review stage at Science including two Pennsylvania (Drexel and University of Pennsylvania) and two Massachusetts (both Harvard) based laboratories. Further, a paper is being prepared for submission to Analytical Chemistry

based on the work of Drs. Frey and Snyder on a methodological extension allowing novel measurements relating to metabolic pathway analysis using the data from projects funded by this grant. Directly resulting from the progress in Aim 1, three grant proposals have been written; one internal grant, one R21 proposal, and one subcontract as part of an R01.
As per the anticipated timeline in the proposal, work on aim 2 has not begun as of the timing of this progress report, and future directions will not be discussed as per directions of this progress report.

Specific Aim 1 Progress:

Progress towards aim 1 has consisted of the completion of three major experiments, all in-line with the original proposal. First, multiple generation of flies were grown on undefined medium supplemented with universally labeled 13C6-glucose. After cold 80:20 (v/v) methanol: water extraction and analysis of the molecular isotopic envelopes using liquid chromatography-high resolution mass spectrometry (LC-HRMS), by targeted (Xcalibur) and untargeted methods (13XCMS), insufficient and sporadic labeling was observed (data not shown). This was attributed to the variable and undefined nature of growth medium for Drosophila which consists of primarily of yeast extract. Due to the nature of isotopic labeling experiments with offer standard deviation of a few percentage points of less of isotopic labeling under controlled conditions, this finding was not acceptable to proceed with the project without rectification.

Logically, the second experiment was to rectify this deficiency of the first experiment. To pursue this, a recently published methodology (Piper lab, 2014, Nature methods) which provided defined growth media for Drosophila was adapted for use with isotopic tracers by replacement of key substrates with isotopically labeled analogs, or their unlabeled counterparts as a control. Glucose was again used as the first test substrate, due to the ability to track glycolytic intermediates, and the utilization of glucose derived carbon throughout the fly. Three different growth periods were used to define labeling. The first was adult flies labeled only in adult life by switching to labeled media, and then allowed to procreate producing larva that were grown completely on labeled media. This second generation of flies was then grown to adult hood and split into three groups, with one extracted and stored at -80, one returned to unlabeled media to act as a rescue, and one passaged further on labeled media to continue labeling. This process has now been repeated for 3 generations, with labeling observed in a subset of molecules for the first and second labeled flies. In all extractions, protein extract has been stored at -80 for batch analysis of histone modification, as attempting these assays piecemeal would be a significant cost and inefficient use of grant resources.
With the success of labeling, the third experiment for Aim 1 has been started, with labeling from amino acid, and fatty acid sources exactly as planned in the original proposal. Thus, despite the unanticipated issue of undefined fly growth conditions, we have adapted and progressed the project to realign with the original aims.

Specific Aim 2 Progress:

Completion of growth optimization and analytical conditions have been completed to allow progress on aim 2. As it was not possible to select metabolite replacement without the

experiments in Aim 1 this is a logical and efficient use of time and resources. This is in line with our original timeline of the experiments, as this was aim was anticipated to occur during the final months of the project.

Tables and figures.

Table 1. Labeling conditions tested to date pursuant to Aim 1.

Substrate	Label	Generations Labeled	LC-HRMS Analysis
D-Glucose	U-$^{13}C_6$	3	Y
L-Leucine	U-$^{13}C_5{}^{15}N_1$	1	N
Palmitic Acid	U-$^{13}C_{16}$	1	N
Sodium Propionate	U-$^{13}C_3$	1	N

Table 2. High quality spectral features with isotopic patterns shifted by glucose labeling in the F1 generation grown from larva to early adult on 13C6-glucose containing media. LC-HRMS data was analyzed by X13CMS, then sorted in python to provide high abundance features (signal intensity greater than 10^5 counts) and plotted as mz and retention time pairs.

mzmed	rtmed	mzmed	rtmed	mzmed	rtmed	mzmed	rtmed	mzmed	rtmed	mzmed	rtmed	mzmed	rtmed	mzmed	rtmed
211.1336	22.0	229.1444	22.0	243.1967	29.0	277.2175	34.6	317.1398	3.6	349.0455	2.2	391.1259	8.7	632.2056	2.1
213.1404	22.1	229.1809	27.6	244.1636	24.1	278.2208	34.6	317.1398	3.9	350.0491	2.2	393.1215	8.7	634.1979	2.2
213.1856	33.6	229.2078	34.5	245.1671	24.1	279.1638	32.9	319.1354	3.6	351.0536	2.1	399.3121	30.9		
215.1034	4.8	230.1843	27.6	245.2034	32.1	279.2244	34.6	319.1355	3.9	359.1506	3.9	400.1110	5.5		
215.1147	2.8	230.2112	34.5	245.2034	29.2	280.1671	32.9	328.0457	11.5	360.1540	3.9	400.3156	30.9		
215.1650	26.2	231.1498	22.1	245.2035	29.0	282.0847	8.9	329.0491	11.5	363.1435	3.9	403.1101	5.5		
215.1651	25.9	231.1877	27.6	253.2174	34.9	283.0688	3.9	336.2550	33.9	367.2164	33.7	432.1987	28.8		
215.1923	33.6	231.2147	34.5	254.2206	34.9	284.0721	3.9	337.2584	33.9	368.2197	33.7	433.2021	28.8		
216.1068	4.8	236.0788	12.5	255.2239	34.9	284.0902	8.9	338.2707	34.7	369.1407	8.8	447.2524	34.3		
216.1180	2.8	236.0788	6.2	256.2259	34.9	285.0935	8.9	339.0708	2.9	372.1166	2.7	449.2585	34.3		
217.1215	2.8	237.0645	15.2	257.1761	26.2	286.0982	8.9	339.2737	34.7	372.1509	8.8	454.1908	3.6		
217.1717	26.2	237.0822	6.2	257.2305	34.9	293.1147	4.0	341.0678	2.9	373.1200	2.7	454.1909	3.9		
217.1717	25.9	238.0678	15.2	259.1830	26.2	294.1180	4.0	341.1093	2.2	374.1236	2.7	456.1878	3.9		
223.1701	32.2	238.0843	12.5	267.0738	8.5	296.1003	11.1	342.1126	2.2	382.2605	31.8	458.1865	3.9		
224.1735	32.2	238.0843	6.2	269.0790	8.5	297.1036	11.1	343.1132	2.2	383.1148	10.3	458.1866	3.6		
225.1857	33.2	239.0889	6.2	269.2126	30.3	310.2392	33.4	344.1166	2.2	383.2638	31.8	470.2166	3.9		
227.1923	33.2	243.1603	24.1	270.0824	8.5	311.1763	34.9	346.0628	12.2	384.1181	10.3	472.2122	3.9		
227.2014	34.5	243.1966	32.1	270.2160	30.3	311.2425	33.4	347.0401	2.2	384.2674	31.8	483.1840	2.6		
228.2046	34.5	243.1967	29.2	271.0870	8.5	312.1795	34.9	348.0803	12.2	386.1142	10.3	484.1874	2.6		

Research Project 9: Project Title and Purpose

In Vivo Targeting of ERK1/2 Map Kinase Signaling in Dopaminergic Neurons –
Psychostimulants such as cocaine and amphetamine trigger their rewarding and addictive effects by an acute elevation of extrasynaptic levels of dopamine (DA). Current treatments for psychostimulant use disorders are acknowledged to be ineffective and therefore finding better treatment options is imperative. The research outlined in this project is designed to characterize mediators of adaptive dopaminergic changes that occur during psychostimulant exposure that will permit the identification of novel therapeutic treatment opportunities. The goal of this project is to determine the role of ERK1/2 Map kinase signaling in dopaminergic neurons *in vivo* in the regulation of cocaine-associated DA homeostasis.

Anticipated Duration of Project

1/1/2015 – 6/30/2016

Project Overview

We propose to use unique genetic tools that enable *in vivo* modulation of ERK1/2 map kinase signaling in dopaminergic cells. We have generated adeno-associated viral (AAV) vectors with Cre recombinase (Cre)-dependent expression of transcripts designed to increase or reduce the expression of the ERK1/2 phosphatase MKP3. These viral vectors will be employed in combination with transgenic rats that express Cre in tyrosine hydroxylase (TH)-positive cells. The AAVs will be injected into the ventral tegmental area (VTA) to enable both up- and down-regulation of ERK1/2 signaling in dopaminergic neurons. Our goal is to use this model to further our understanding of the role *in vivo* of ERK1/2 in dopaminergic cells in regulating dopamine (DA) neurotransmission. We have in support of this approach in preliminary experiments demonstrated that employing these tools to modulate ERK1/2 activity specifically in dopaminergic cells in living animals' results in changes in cocaine-associated behaviors.
Aim 1: Examine *in vivo* effects of ERK1/2 signaling on dopaminergic proteins: We will use the viral expression tools to study how modulating ERK1/2 signaling in dopaminergic cells *in vivo* affects dopamine transporter (DAT), calcium channel expression and subcellular localization. To establish changes in calcium channel expression immunohistochemistry will be performed. To assess changes in expression and surface expression of DAT we will perform cell-surface biotinylation assays on striatal synaptosomal preparations.
Aim 2: Investigate the role of ERK1/2 signaling in regulating dopaminergic release and uptake using fast scan cyclic voltammetry (FSCV): We will employ FSCV to study the effects of modulating ERK1/2 signaling in dopaminergic cells on *in vivo* DA release and reuptake. We will focus on changes in release and reuptake properties including reuptake sensitivity to cocaine. FSCV is a versatile technique that confers sub second information about neurotransmitter release and uptake in real-time. Rats will be implanted with a jugular catheter, a stimulating electrode in the VTA, and a carbon-fiber electrode in the Nucleus Accumbens to measure baseline and cocaine-induced changes in DA release and uptake.

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Expected Research Outcomes and Benefits

This project will allow labs with different expertise to use innovative methods to work together on a goal that is highly significant: achieve a better understanding of the role of ERK1/2 signaling in cocaine addiction with the ultimate goal of providing new insights and identify novel targets that can have therapeutic relevance for the treatment of cocaine use disorder. By combining our respective very different research strengths the study will be enhanced in a synergistic way that cannot be achieved by us individually. Combining biochemical knowledge with ex vivo functional studies will position us in a unique position that will enable innovative research on understanding cocaine use disorder. Cocaine abuse is an enormous problem for the American society causing suffering and harmful consequences to people of all ages. No effective pharmacological treatment exists for this disorder and research that can address this lack of therapeutic options is therefore of great importance. We believe our study is a step towards this goal by exploring downstream molecular mediators of ERK1/2 signaling that establish physiologic adaptations following repeated cocaine use. Such knowledge will be critical for identifying specific molecular mechanisms of neuroplastic changes leading to cocaine use disorder. Successful completion of this project will as a consequence lead to novel insights into signaling systems that mediate adaptive cellular and molecular changes within dopaminergic neurons involved in the development of cocaine use disorder. It is anticipated that the experiments described in this application will identify novel targets that mediate cocaine-associated behaviors and potentially lead to novel therapeutic avenues for treating this disorder.

Summary of Research Completed

Specific Aim 1 Progress:
Transgenic colony:
To enable the proposed research we have established a colony of transgenic rats specifically expressing Cre recombinase in tyrosine hydroxylase positive dopaminergic and noradrenergic neurons (TH-Cre rats). We have been successful in starting a breeding scheme for this purpose. This was initiated in the beginning of the project year and we have at the current moment obtained two rounds of litter. The initial two male TH-Cre animals were obtained from an external source (RRRC) and these animals therefore were required to go into quarantine and they were not available for initiating the transgenic colony until the end of February 2015.

Immediately following release a breeding scheme was initiated. The first litter was obtained by the end of March 2015.

Genotyping of litters:
With tissue available from the first litter we have optimized a genotyping protocol for robust detection of hemizygous rats that has one copy of the Cre allele. See figure 1. From the first litter of 36 pups we identified the expected number of hemizygous males and females (17 positive). The second litter of pubs was recently born and has now been weaned. From this litter of 40 pubs we identified 20 Cre-positive animals.

Viral injections:
We have moved forward with the 6 transgenic males from the first litter and in the beginning of June 2015 successfully injected them with AAV-MKP3 virus into one hemisphere of the VTA and a control virus that express GFP in a Cre-dependent manner into the other hemisphere

Biochemical assays:
We are now in the process of setting up and optimizing biochemical assays. These include cell surface biotinylation of synaptosomal preparations and immunohistochemistry of calcium channel expression. The optimal antibodies for these experiments have been determined. For DAT expression we will use a Millipore antibody (Millipore MAB369) and for the calcium channel expression we will use antibodies from Alomone labs (Cav1.2:ACC-003, Cav 2.2:ACC-002).

The following methods have been determined to be optimal for these procedures:

Immunohistochemistry: Rats are rapidly anesthetized before intracardiac perfusion with heparinized saline followed by 4% (w/vol) paraformaldehyde. Brains are post fixed overnight in the same solution and stored at 4°C; incubated in 30% sucrose in 0.1 m phosphate buffer for cryoprotection, and subsequently frozen in dry ice. Brains from paraformaldehyde perfused animals will be cut in 25 µm thick sections. Free-floating sections are incubated with antibodies overnight at 4°C. Sections are subsequently incubated with secondary fluorescent antibodies. Sections are mounted on glass slides and cover slipped. Images from each region of interest are obtained using fluorescent or confocal microscopy. Expression of viral constructs is verified by EGFP expression.

Synaptosomal surface biotinylation: The bilateral striata are rapidly dissected on ice and homogenized in ice-cold phosphate buffer containing 0.32 M sucrose. The tissue homogenate is centrifuged at 1000 g for 12 min at 4 °C, and all subsequent centrifugations also occur at 4 °C. The supernatant is centrifuged at 12,500 g for 15 min to isolate the synaptosomal P2 pellet. The tissue is resuspended at 100 mg wet weight/ml in Krebs assay buffer. The tissue is centrifuged at 8000 g for 4 min at 4 °C, and the resulting pellet is resuspended at 100 mg wet weight/ml in phosphate buffered saline (PBS)/Ca^{2+}/Mg^{+} buffer containing 2 mg/ml sulfo-NHS-biotin and incubated for 1 h at 4 °C with gentle agitation. The biotinylation reaction is terminated by addition of 1 ml 1.0 M glycine in PBS/Ca^{2+}/Mg^{+} buffer for 10 min on ice. Tissue is then centrifuged at 8000 g for 4 min, and the pellet is resuspended in 1 ml of 0.1 M glycine in

PBS/Ca^{2+}/Mg^{+} buffer. This step is repeated twice, followed by incubation of tissue in 1 ml of 0.1 M glycine in PBS/ PBS/Ca^{2+}/Mg^{+} buffer for 30 min at 4 °C with gentle agitation. Following incubation, tissue is centrifuged at 8000 g for 4 min followed by three washes in 1 ml of PBS/Ca^{2+}/Mg^{+} buffer. The final pellet is resuspended in 300 µl of 1% Triton X-100 buffer containing protease inhibitors and subjected to 2–4 s of sonication, before 30-min incubation at 4 °C with gentle agitation. Tissue is then centrifuged at 20,000 g for 30 min. The supernatant is incubated with 100 µl of neutravidin beads for 15–16 h at 4 °C with gentle agitation. Samples are then centrifuged at 17,700 g for 4 min. The supernatant is discarded, and the biotinylated protein/avidin bead complex is washed three times with 1 ml of Triton X-100 buffer. Beads are then incubated with 100 µl of sample buffer. The samples are analyzed by SDS-PAGE immunoblotting using a DAT specific antibody (Millipore MAB369).

AAV constructs for cre-dependent MKP3-silencing:
To achieve Cre-dependent silencing of MKP3 we are pursuing and optimizing MKP3-silencing constructs. We have produced 4 different miR30-based targeting constructs that are being tested for silencing of MKP3 in *in vitro* cell culture experiments to determine the most efficacious construct. The most efficacious construct will be used to produce AAV particles to produce *in vivo* silencing of MKP3 expression in a Cre-dependent manner in the TH-Cre rats.

Specific Aim 2 Progress:
Investigate the role of ERK1/2 signaling in regulating dopaminergic release and uptake using fast scan cyclic voltammetry (FSCV): We will employ FSCV to study the effects of modulating ERK1/2 signaling in dopaminergic cells on *in vivo* DA release and reuptake. We will focus on changes in release and reuptake properties including reuptake sensitivity to cocaine.
FSCV is a versatile technique that confers subsecond information about neurotransmitter release and uptake in real-time. Rats will be implanted with a jugular catheter, a stimulating electrode in the VTA, and a carbon-fiber electrode in the Nucleus Accumbens to measure baseline and cocaine-induced changes in DA release and uptake.

Following successful completion of the experiments in Aim 1 that will establish if our stereotaxic injections are successful in achieving specific expression of our constructs in the dopaminergic neurons of the VTA we will use the same parameters for the proposed experiments in Aim 2. Our labs are continuously performing the assays that are described for this aim and we therefore expect to be able to perform these assays on our experimental animals without much optimization.

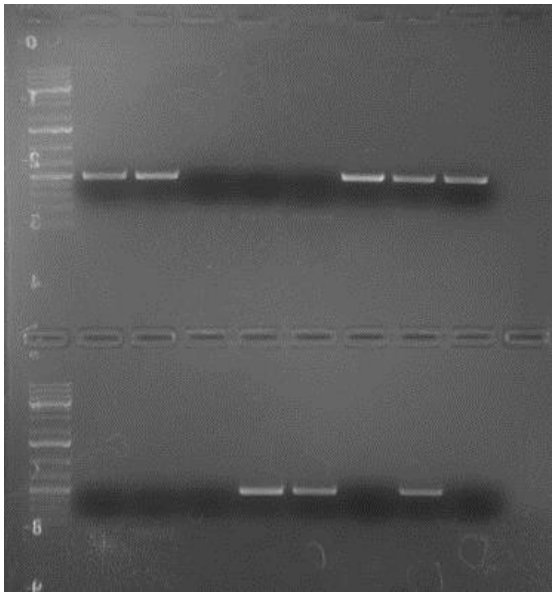

Figure 1. Genotyping of rat pubs by performing PCR on DNA isolated from the tail snips of individual pups. PCR with Cre specific primers produce a specific band of the predicted size only in animals carrying a copy of the Cre recombinase gene.

Research Project 10: Project Title and Purpose

Mechanical Circulatory Assistance for Congenital Heart Disease: Biologically-Inspired Heart Pumps – The treatment of congenital cardiac Fontan anomalies is a formidable and costly challenge for clinical teams caring for patients with congenital heart disease. Surgical and pharmacologic treatment only slows the progression of premature heart failure in these patients. The availability of donor organs is limited given the number of patients in need, and no mechanical blood pump has been specifically developed for Fontan patients. The purpose of this project is to establish the ideal biofluid dynamic mechanical circulatory support conditions for Fontan patients. The goal of this project is to inform the new development of a novel, biologically inspired, axial flow blood pump for these patients as a viable treatment option in their clinical management.

Anticipated Duration of Project

1/1/2015 – 12/31/2016

Project Overview

The treatment of congenital cardiac anomalies is a formidable and costly challenge for clinical teams caring for patients with congenital heart disease. Patients with dysfunctional single ventricle (Fontan physiology) utilize healthcare resources disproportionate to their numbers. Those fortunate to survive surgical palliation contend with morbidity and lifelong physical complications. Pharmacologic therapy and surgical modifications have improved patient

outcomes, but only slow the progression of premature congestive heart failure. The lack of therapeutic alternatives for these patients motivates the development of blood pumps as a bridge-to-transplant, bridge-to-recovery, and bridge-to-hemodynamic stability.

Thus, we hypothesize that rotational blood flow, delivered by an axial flow blood pump, will more effectively assist the venous circulation in Fontan patients by controlling and directing the momentum imparted from the generated vortex and by mimicking actual blood flow conditions in the cardiovascular system. The purpose of this project is to determine the optimal biofluid dynamic conditions in a new, biologically inspired, blood pump for Fontan patients.

Having a proven track record of success in this field, we have generated data through numerical modeling, prototype hydraulic evaluation, and laser flow measurements to demonstrate the feasibility of our project. Building upon this data, we propose to establish optimal biofluid dynamic characteristics in patient-specific Fontan physiology by computational analyses using specified boundary conditions of post-swirl blood flow during the cardiac cycle and respiration, and to investigate the benefits and limitations of rotational blood motion by conducting experimental flow visualization studies.

This project will elucidate the blood flow characteristics for mechanical circulatory support of dysfunctional Fontan physiology and will inform the translational design of a new medical device for these patients. The interdisciplinary expertise of our team places us in an excellent position to assess the integration of mechanical support into the Fontan, thereby addressing a significant human health problem.

Specific Aims:
Aim 1: Establish optimal biofluid dynamic characteristics of mechanical circulatory support (MCS) in the Fontan physiology.
Aim 2: Determine the benefits and limitations of rotational blood motion by conducting experimental flow visualization studies of MCS in patient specific Fontan physiology.

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Expected Research Outcomes and Benefits

A blood pump specifically designed to augment flow from the great veins through the lungs would ameliorate the poor physiology of the high-risk Fontan circulation. No pump has been developed, and currently no viable therapeutic alternative exists. To address this substantial need, this project will elucidate the appropriate blood flow characteristics for mechanical circulatory support of dysfunctional Fontan physiology and will inform the translational design of a new medical device for Fontan patients. We have assembled an ideal multidisciplinary research team who will contribute jointly and collaboratively through their respective disciplines and expertise to develop this blood pump for patients with a Fontan physiology. Based on the study findings, we will pursue additional extramural funding to government agencies and foundations. A priority is to document new intellectual property and, when appropriate, to publicly present and publish our findings. The PI has a long history of promoting education and research in the science, technology, engineering and mathematics disciplines; she will make contact with local schools in order to coordinate a student visit to the BioCirc Lab in Biomedical Engineering and the Cardiovascular Lab in Cardiothoracic Surgery during next summer in order to learn about this project and medical device development that lies at the intersection of engineering and medicine. This project will advance the newly emerging field of mechanical Fontan by assisting and contributing to the development strategy of an exciting new therapeutic tool that will revolutionize the treatment approach for Fontan patients.

Summary of Research Completed

Specific Aim 1 Progress:
This study involves the numerical analysis of the mechanical cavopulmonary assistance using a patient specific anatomic configuration of the Fontan. Mechanical assistance is modeled in two ways: 1) an axial flow blood pump located in the inferior vena cava (IVC); and 2) an axial flow blood pump located in both the IVC and superior vena cava (SVC) for a dual-support scenario. This is the first study to investigate the impact of pre- and post-swirl dynamics in the TCPC due to mechanical circulatory assistance. The simulations included an assessment of the pressure-flow characteristics of the axial blood pump, hydraulic energy calculations for the cavopulmonary circulation with and without pump support, and a blood damage analysis. Five computational models have been generated and analyzed, one computational model remains to be analyzed, and we evaluated the performance for all of the pump configurations for 6 pulmonary arterial mean pressures (9, 12, 15, 18, 21, and 24 *mmHg*), pump rotational speeds of 1250-2500 revolutions per minute (RPM), and flow rates ranging from 1-4 *L/min*.

Axial Flow Blood Pump Design: Fig. 1 displays the conceptual design of the cavopulmonary assist device. It is designed for percutaneous positioning in the IVC, SVC, or extra-cardiac conduit (ECC) of the single ventricle physiology. The outer protective cage has radially arranged straight filaments and a set of diffuser blades that are mounted at the outlet. The pump consists of an impeller with three uniquely designed blades having characteristic angles that are appropriate to achieve the desired operating range. Pump rotation is induced through a drive cable-fluid seal combination with a port to supply a dextrose solution as lubrication between cable and polyurethane outer cover, as well as to flush the fluid seal of any accumulated blood elements.

The pump is designed to generate flow rates of 1-4 *L/min* with pressure rises of 2-25 *mmHg* for 3000-8000 RPM. It will also provide 4 weeks of temporary mechanical circulatory support with replacement possible for prolonged support.

Computational Models: *Anatomic Configuration*: Through an investigational review board (IRB) approved retrospective study of patient data; we selected 2-D magnetic resonance images (MRI) of a Fontan circulation. Using these images, a patient-specific anatomic model was created by transforming the MRI data set into a 3-D CAD solid body. We employed the software *MIMICS* (Materialise, Leuven, Belgium) to generate a 3-D point cloud mesh from the patient's MRI data set and to produce the solid body model in the computer-aided-design software. Connecting vessel sections of the TCPC were extended for CFD analysis with tapering of vessels to ellipse forms. Placement of the blood pump was in the IVC and SVC. Fig. 2 illustrates the patient specific TCPC model generation with pump support.

Cavopulmonary Assistance Models: Fig. 3 demonstrates the new computational models of mechanical cavopulmonary assistance scenarios that were created for this study using the computer-aided design (CAD) software SolidWorks (SolidWorks, Concord, MA, USA). The models consist of the anatomic TCPC with: A) TCPC without pump support; B) an axial blood pump in the IVC rotating in the clockwise (CCW) direction; C) an axial blood pump in the IVC rotating in the counterclockwise (CW) direction; D) an axial blood pump in the IVC and SVC rotating in the CW direction; E) an axial blood pump in the IVC rotating in the CW direction and in the SVC rotating in the CCW direction; F) an axial blood pump in the IVC and SVC rotating in the CCW direction; and G) illustration of CCW and CW impeller and diffuser blade orientations. The following numerical studies have been completed to-date: A, B, C, D, and F.

Fluid Dynamic Solver: In this study, we utilized ANSYS CFX 12.1 software (ANSYS Incorporated, Canonsburg, PA) to simulate the fluid dynamics through the new pumps and anatomic Fontan geometry. The program, CFX-Mesh, was employed to generate the tetrahedral mesh. After completion of mesh generation, the computational flow model was implemented in the Reynolds-Averaged Navier Stokes fluid solver, ANSYS CFX 12.1. The k-ε turbulence model was selected based on the successful correlation of bulk performance parameters (i.e. pressure-flow characteristics) in geometrically similar prototypes from previous work. A grid density and a convergence study were performed to ensure mesh quality, where incremental adjustments to the grid size were made until the performance results deviated less than 3%.

Boundary Conditions: The no-slip boundary condition was applied to the stationary walls of the models for these steady flow simulations. In the stationary reference frame, the TCPC configuration, catheter, cage of filaments, and diffuser blade surfaces were defined as stationary boundaries. The impeller of the pump rotor, however, was specified to be in the rotating reference frame in accordance with the impeller blade orientation. The frozen rotor interface connected regions of differing reference frames and maintained flow properties without circumferential averaging. A uniform mass inflow rate or cardiac output was specified for each simulation based on a 60%/40% flow split between the IVC and SVC, as appropriate for older Fontan patients. The pump rotational speeds were evaluated at 1250-2500 RPM. The outlet boundary conditions, such as the left and right pulmonary arteries (LPA and RPA), were defined

to have static and equal mean pressures of 9, 12, 15, 18, 21 and 24 *mmHg*. All of the vessel walls were modeled as rigid tubes. A constant viscosity value of 0.0035 kg/m*s and fluid density of 1,050 kg/m^3 were used.

Blood Damage Estimation: We will perform a blood damage analysis on all of the CFD models to examine the potential for hemolysis and thrombosis. This damage analysis has been widely employed as a predictive tool in the development of several rotary blood pumps. This approach estimates the 3-D flow field and calculates a scalar stress (σ) as representative of the level of stress experienced by the blood traveling through the pumps and TCPC domain. Fluid streamlines, as indicative of predicted fluid residence times, will be evaluated. Using a power law relationship between the scalar stress level and the exposure time, a blood damage index will be estimated for the selected models. The accumulation of stress and exposure time will be added along the streamlines. The blood damage index is represented as a percentage of the change in hemoglobin levels due to blood trauma divided by the original hemoglobin content. A blood damage index below 2% is the target design requirement.

Energy Gain in the Cavopulmonary Circulation: The addition of a blood pump in the cavopulmonary circulation will impact the overall energetics of the system. We will use a control volume approach to calculate the energy losses through TCPC configuration with and without the pump. This technique is a common approach used when considering surgical optimization of the TCPC, and the power dissipation or rate of energy change in the cavopulmonary configuration can be estimated.

Specific Aim 2 Progress: To enable the study of biomimetic vortical / rotational blood motion imparted by the new designs of mechanical circulatory support (MCS) devices at Drexel University, the Cardiothoracic Surgery Research 'Heart Lab' has constructed a mock circulatory loop with an TCPC, complete with passive and active flow capabilities. The diagram in Fig. 5 provides a broad conceptual overview of the single ventricle physiology, incorporating the 'surgically palliated' TCPC section that is central to the simulation of powered/ active Fontan circulation. This schematic depicts the lumped systemic and pulmonary vascular beds in form of SVR and PVR respectively. Relative pressures shown in the diagram are representative venous pressure values in the absence of an intact 'right pump'.

A mock circulatory loop has been constructed to accommodate the MRI-acquired, patient specific TCPC model (Fig. 6B). As shown in Fig. 6A, the flow loop consists of a supply reservoir (i.e. venous reservoir), the TCPC model, a collection reservoir, and a supply / return pump. Flow through the circuit is driven by the elevated reservoir by the supply pump drawing from the collection reservoir and can be augmented (actively). Several occluders and valves are incorporated to enable control over the 'vascular / system load', and consequently volume flow rate in individual conduits (e.g. SVC, IVC, RPA, LPA). Fig. 6A depicts and locates the desired positioning of the rotameters and pressure measurement sites. The prototype pump is connected to the IVC such that the flow can be electively diverted to simulate an active Fontan flow configuration. This mock circulatory loop contains silicone tubing, making it amenable for ultrasonic flow visualization. The full in-lab extracorporeal setup is shown in Fig. 7.

The experimental test conditions (both passive and active flow circulations) are simulated and studied using a glycerol-saline (44:56) blood analog seeded with ~20µm Sphericel® hollow glass microspheres (Potters Industries LLC).Load resistances are used to selectively program and achieve a SVC: IVC split flow distribution of 40:60, and 50:50 for RPA: LPA division. Studies for the passive Fontan will be undertaken with the following conditions: total flow rate (i.e. cardiac output) of 3, 3.5, and 4 L/min; average pulmonary arterial pressures of 12, 16, 18 and 22 mmHg. Mechanically assisted active Fontan circulation will be studied using clockwise and counter-clockwise impeller configurations at pump speeds of 2000-5000 RPM. This will allow imaging of helical flow formation by the computer-designed and optimized impeller localization, including the post-swirl dynamic flow formation.

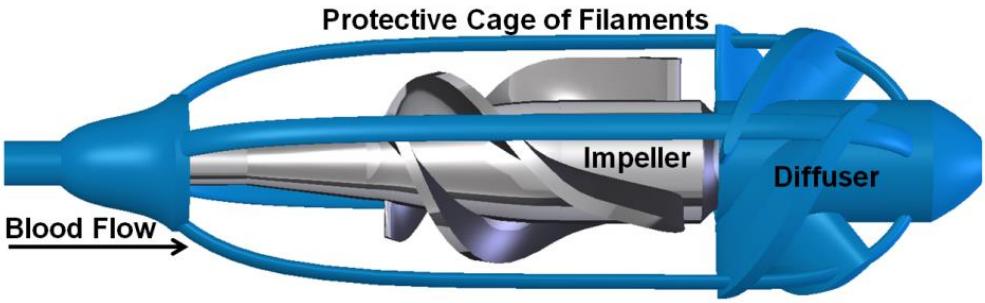


Figure 1: Axial Flow Blood Pump for Mechanical Cavopulmonary Assistance. Design consist a catheter, protective cage of filaments, impeller blade set, and diffuser blade set.

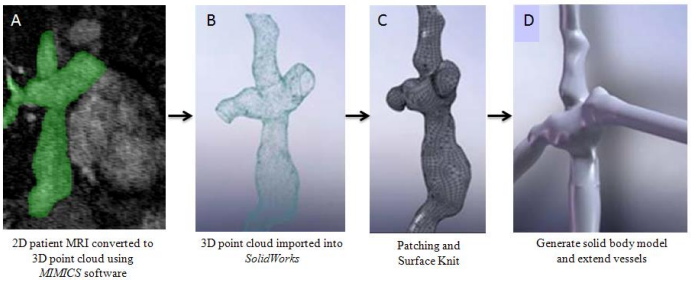


Figure 2: Generation of the Patient-specific Anatomic Model. A. 2D patient MRI converted to 3D point cloud in MIMICS, B. Smooth point cloud mesh imported into SolidWorks; C. Surface knit to solid body; D. Vascular extensions to the solid body TCPC.

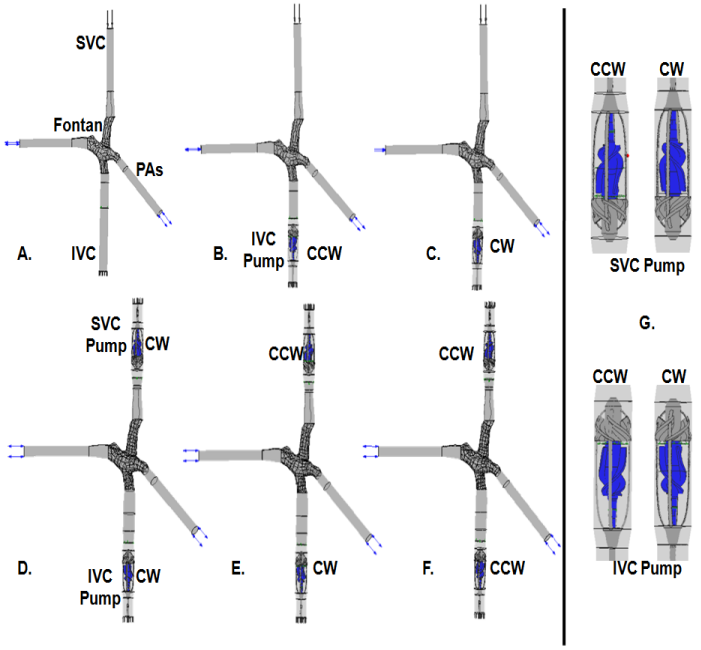


Figure 3: Numerical Models of Mechanical Cavopulmonary Assistance with Patient-Specific Fontan Model. A) TCPC without pump support; B) an axial blood pump in the IVC rotating in the clockwise (CCW) direction; C) an axial blood pump in the IVC rotating in the counterclockwise (CW) direction; D) an axial blood pump in the IVC and SVC rotating in the CW direction; E) an axial blood pump in the IVC rotating in the CW direction and in the SVC rotating in the CCW direction; F) an axial blood pump in the IVC and SVC rotating in the CCW direction; and G) illustration of CCW and CW impeller and diffuser blade orientations.

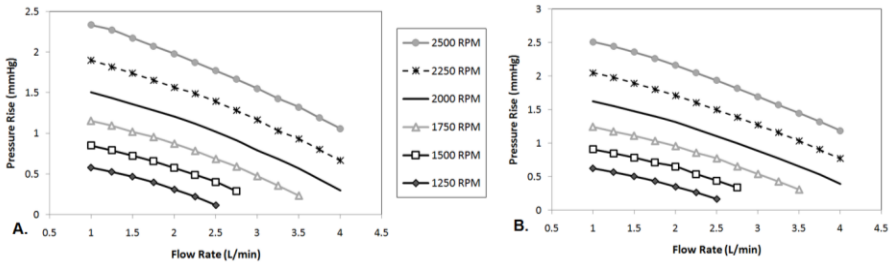


Figure 4: Pressure Generation for CCW and CW Configured Cage and Pump Placed into IVC, a Range of Rotational Speeds, Flow Rates of 1-4 L/min, and Equal Pulmonary Arterial Pressures of 15 mmHg. A) CCW configured pump in the IVC; B) CW configured pump in the SVC. Initial results of computational analysis.

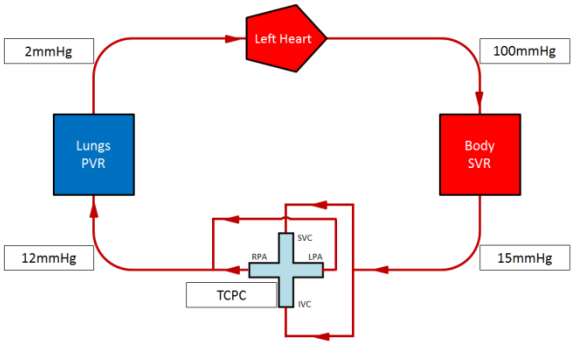
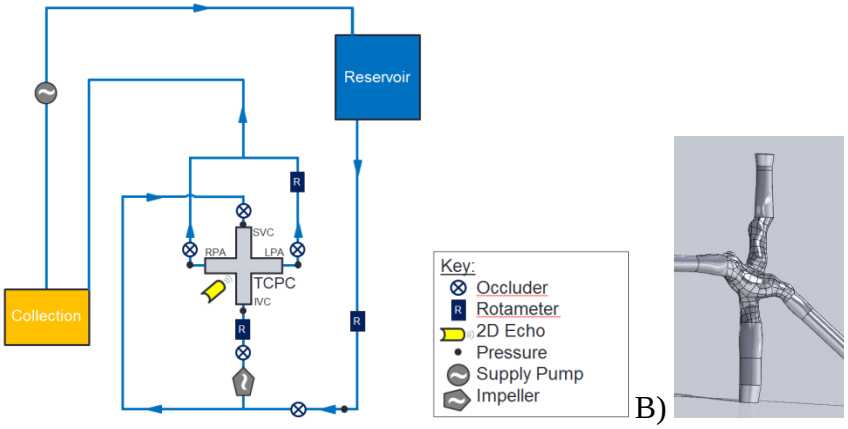


Figure 5: Conceptual model of a single ventricle (Left-Heart) circulatory system physiology. The Fontan vascular anastomosis consists of a Total Cavopulmonary Connection (TCPC). The arterial (lumped) vasculature is represented by SVR (systemic vascular resistance), and pulmonary (lumped) vasculature by PVR (pulmonary vascular resistance).



A) B)

Figure 6 :(A) Schematic representation of the bench-top mock circulatory circuit depicting the location of relevant ‘control’’ components (e.g. occluders, flow rotameters, reservoirs) and pressure measurement ports. Silicone tubing (with acoustical impedance similar to fluid) serves as a coupling / interface to enable visualization using Doppler and 2D color ultrasound.(B) An MRI-scanned patient specific TCPC prototype was rendered and fabricated as 3D-physical model and incorporated into the mock circulatory loop.

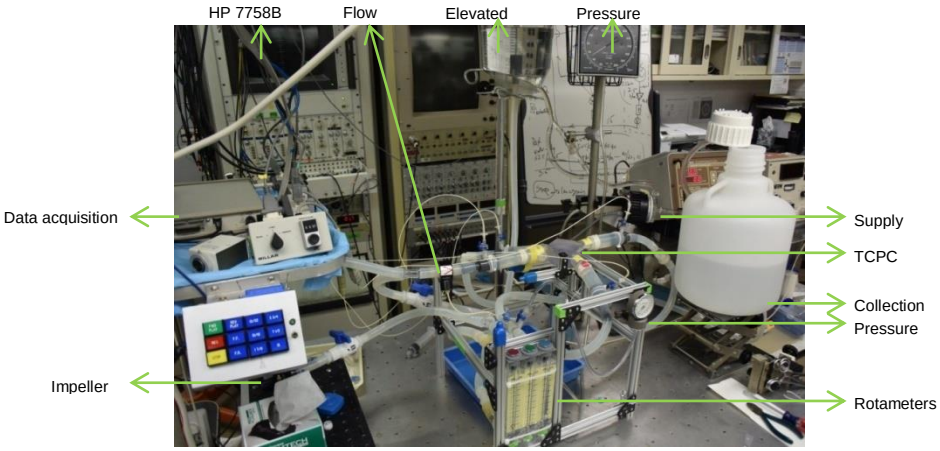


Figure 7: A bench-top mock circulatory setup was designed and assembled within the Cardiothoracic Surgery 'Heart Lab'. The identified fluid-circuit components include the pump-head inflow and outflow reservoirs, TCPC, electromagnetic flow meter, flow rotameters, supply pump, impeller pump, pressure transducers and gauges.

Research Project 11: Project Title and Purpose

Direct Role and Mechanism of Activation and Expression of BKCa Channel in Cardioprotection from Ischemia-Reperfusion Injury. – Large conductance Ca2+ and voltage-activated potassium channels (BK_{Ca}) were discovered in cardiac mitochondria where they were shown to be involved in cardioprotection from ischemia-reperfusion (IR) injury by activation with pharmacological agents (NS1619). Outstanding questions are, does BK_{Ca} play a direct role in cardioprotection or is it via non-specific effect of pharmacological agents used? And, what is the mechanism of BK_{Ca}-mediated in cardioprotection? The proposed project will facilitate establishment of the direct role of BK_{Ca} in cardioprotection from IR injury and provide a possible mechanism involved in BK_{Ca} -mediated cardioprotection.

Anticipated Duration of Project

1/1/2015 – 12/31/2016

Project Overview

Increasing evidence suggests that mitochondrial inner membrane K^+ channel sensitive to voltage and Ca^{2+} (mitoBK_{Ca}) is directly involved in protecting the heart from ischemia-reperfusion (IR) injury. One of the putative protective mechanisms of mitoBK_{Ca} is modulation of mitochondrial permeability transition pore (mPTP) by decreasing the reactive oxygen species (ROS) as well as increasing the mitochondrial calcium retention capacity (CRC). However, all studies implicating BK_{Ca} in cardioprotection have profoundly relied on usage of pharmacological agents (agonists and antagonists) whose specificity has been dubious. Although it is known that pharmacological-activation of BK_{Ca} is essential for cardioprotection, there is limited information available on the physiological role of BK_{Ca} in mitochondria as well as cardioprotection. We propose to test the hypotheses that: 1) cardiac BK_{Ca} plays a direct role in cardioprotection; and, 2) BK_{Ca} plays a direct role in mitochondrial physiology which contributes to its cardioprotective role. Our preliminary data show that: 1) mitoBK_{Ca} is encoded by a *Kcnma1* gene, and a DEC splice variant governs its mitochondrial localization; 2) BK_{Ca} ablation prevents the cardioprotective effect of NS1619 (BK_{Ca} agonist); 3) activation of BK_{Ca} increases CRC, hence delaying the opening of mPTP, 4) Paxilline (BK_{Ca} antagonist) reduces CRC in freshly isolated mitochondria; and 5) activation of BK_{Ca} reduces ROS generated by complex I of electron transport chain (ETC). Our preliminary data supports our hypothesis that cardiac mitoBK_{Ca}, which is exclusively present in the mitochondria of adult cardiomyocytes, does play a cardioprotective role and this will be tested by an array of multidisciplinary collaborative approach using mouse genetics. We will pursue the following specific aims: 1) To determine the role of BK_{Ca} in cardioprotection from IR injury; and, 2) To establish the direct role of BK_{Ca} in mitochondrial physiology and mechanism in cardioprotection. This program will be important for determining how cardiac BK channels directly play a role in mitochondrial physiology and cardioprotection. *The outcomes of this investigatory project will open the opportunity to study BK_{Ca} at the physiological levels, and advance the cardiac field by defining functional properties of cardiac BK_{Ca} in mitochondria, and in cardioprotection from IR injury.*

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Expected Research Outcomes and Benefits

One of the mechanisms involved in protecting the heart from lack of oxygen like that occurring during heart infarct is thought to be the opening of a mitochondria potassium channel named mitoBK_{Ca}. Here, we propose to unveil the role of cardiac mitoBK_{Ca} in protecting the heart from ischemia and reperfusion injury. By establishing the direct role of BK_{Ca} in cardioprotection and identifying the mechanism of cardioprotection, we can isolate new drug targets for cardioprotection. The results of the current project will allow the progress of cardioprotective medicine and provide new molecular targets for therapeutics.

Summary of Research Completed

Specific Aim 1 Progress:
We have generated BK_{Ca} knock out, BK_{Ca} TG (gain of function) and wild type mice for pursuing aim 1 and 2. We have initiated experiments to study the direct role of BK_{Ca} in cardioprotection.

Ischemia-reperfusion was carried out on hearts isolated from wild type and BK_{Ca} knout out mice (*Kcnma1*$^{-/-}$) (fig 1) as published earlier[1,2]. Hearts were treated with 10μm NS1619 (BK_{Ca}-agonist) or DMSO (vehicle control, 1:1000) for 10 min before ischemia. After global ischemia for 18 mins, hearts were perfused for 60 mins, and during this procedure heart functions were recorded. As illustrated in Fig 1, NS1619 recovered 100% left ventricular developed pressure (LVDP) (Fig 1C) in wild type but not in BK_{Ca} knout out mice (Fig 1D). However, after ischemia *Kcnma1*$^{-/-}$ mice showed reduced LVDP (Fig 1B and 1C). Myocardial infarction (MI) was also measured at the end of reperfusion and it was discovered that NS1619 protects the wild type (Fig 1C') but not *Kcnma1*$^{-/-}$ mice (Fig 1D'). In our ongoing experiments with BK_{Ca} TG mice, we are carrying out the ischemia-reperfusion injury assessment to corroborate our results obtained from pharmacological agents. Initial experiments suggest that BK_{Ca} TG mice do not show abnormal LVDP after ischemia and reperfusion.

Specific Aim 2 Progress:

To understand direct role of BK_{Ca} in mitochondrial physiology to deduce its mechanistic role in cardioprotection from ischemia-reperfusion injury we have carried out experiments with sham (without IR) and injury (with IR) model mice in the first six months of this project.

Mitochondria were isolated from wild type or BK_{Ca} TG mice without ischemia-reperfusion (sham) and resuspended in reactive oxygen species (ROS) buffer (250 mM sucrose, 1 mM EGTA, 1 mM EDTA, 0.15% (w/v) BSA and 20 mM Tris HCl, pH 7.4). ROS was measured from mitochondria using 10 μM amplex red following manufacturer's instructions and protocol published earlier[1-3] using a Hitachi F-2710 fluorescence spectrophotometer at 37ºC. The excitation and emission were set to 560 nm and 590 nm, respectively, and the time resolution was 0.4 seconds. To understand role of individual complexes, specific substrates (5 mM glutamate/ malate for complex I and 3 mM succinate for complex II/III) were used. As shown in fig 2, ROS was measured and normalized to the protein concentration of isolated mitochondria. After 15 min, 1μm paxilline (BK_{Ca} channel blocker) was added to the buffer, and study its effect on ROS generation by electron transport chain (ETC). DMSO was used as a vehicle control. There were no significant differences observed in ROS generated by wild type mitochondria after paxilline either by complex I (Fig 2A) or complex II/III (Fig 2C). Similarly mitochondria prepared from BK_{Ca} TG mice showed no differences in ROS production either from complex I (Fig 2B) or complex II/III (Fig 2D). However, when mitochondrial ROS from BK_{Ca} TG mice were compared with wild type, significant reduction in ROS generated by BK_{Ca} TG mitochondria was observed for both complex I (Fig 2B vs A) and complex II/III (Fig 2D vs C) implicating that an active BK_{Ca} channel can reduce ROS generation. Blocking BK_{Ca} had no significant effect on ROS as reported earlier. This led us to test our hypothesis *that during ischemia-reperfusion injury activation of BK_{Ca} plays a role in cardioprotection.*

To test our hypothesis, we excised hearts after 10 mins of reperfusion and isolated mitochondria from wild type and *Kcnma1*$^{-/-}$ mice. Hearts were treated with 10 μm NS1619 for 10 min (as shown in fig 1) before ischemia. ROS was measured as described above and different substrates for complex I (glutamate/malate) and complex II/III (succinate) were used. As shown in fig 3, ROS generated by complex I is significantly ($p \leq 0.05$) reduced in wild type (Fig 3A and C) but not in *Kcnma1*$^{-/-}$ mice (Fig 3B and C). Complex II/III mediated ROS showed no significant differences in ROS generation either in wild type mice or *Kcnma1*$^{-/-}$ mice (Fig 3 D, E and F).

Our initial results indicate that BK_{Ca} activation directly play a role in cardioprotection from ischemia-reperfusion injury. Earlier we have shown that regulation of mitochondrial calcium is one of the possible mechanisms and now we have additional data to support that modulation of mitochondrial ROS is also involved in BK_{Ca}-mediated cardioprotective role.

Figure 1

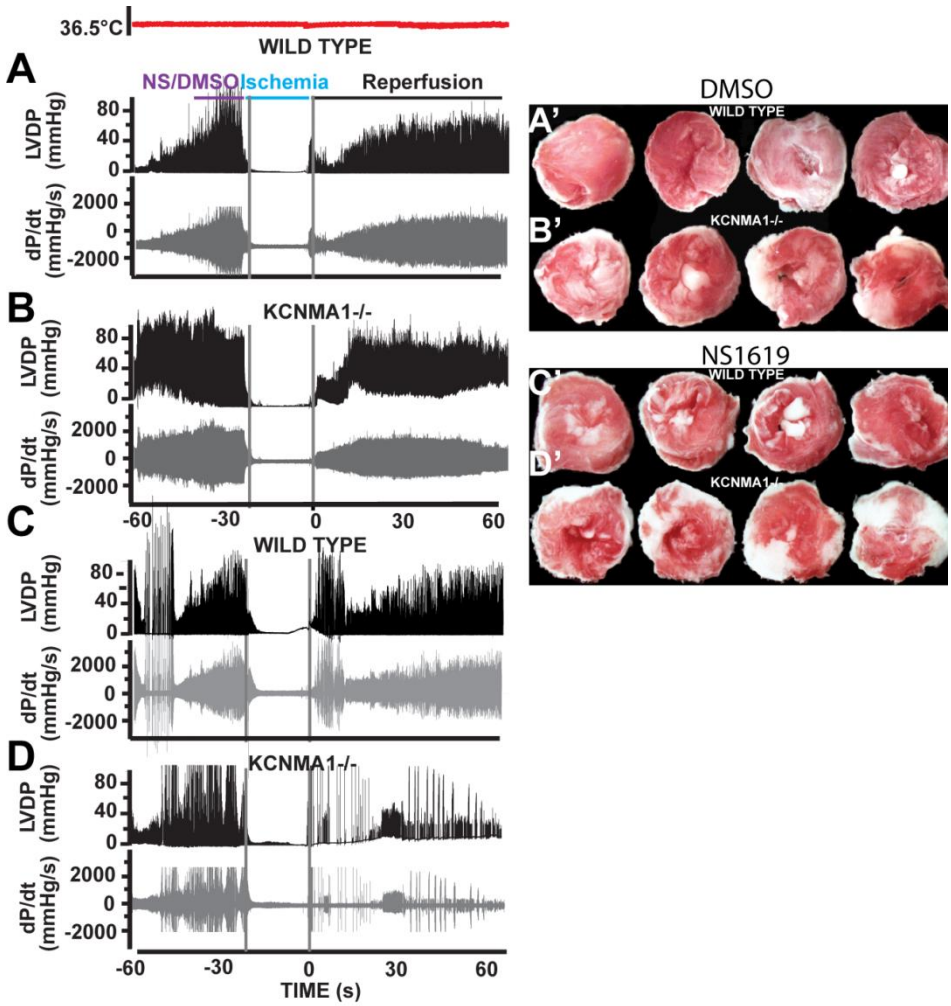

Fig 1. Role of activation of BK_{Ca} in cardioprotection from IR injury. Hearts from wild type and *Kcnma1*$^{-/-}$ mice were perfused at 37ºC. Once hearts were stabilized, they were perfused for 10 mins with either DMSO vehicle control (A and B) or 10 µm NS1619 (C and D). Immediately, flow was stopped and hearts underwent 18 min of global ischemia. After ischemia, hearts were reperfused for 60 mins at 37ºC. Left ventricular developed pressure (LVDP) and dP/dt were measured. After reperfusion hearts were removed and excised into four cross sections. Cardiac sections were stained with TTC at 37ºC for 30 mins. Sections were imaged. Infarction is reduced in hearts treated with NS1619 in wild type (C') and not with DMSO (A'). *Kcnma1*$^{-/-}$ mice showed no protection by NS1619 implicating that expression of BK_{Ca} is essential for cardioprotection.

Figure 2

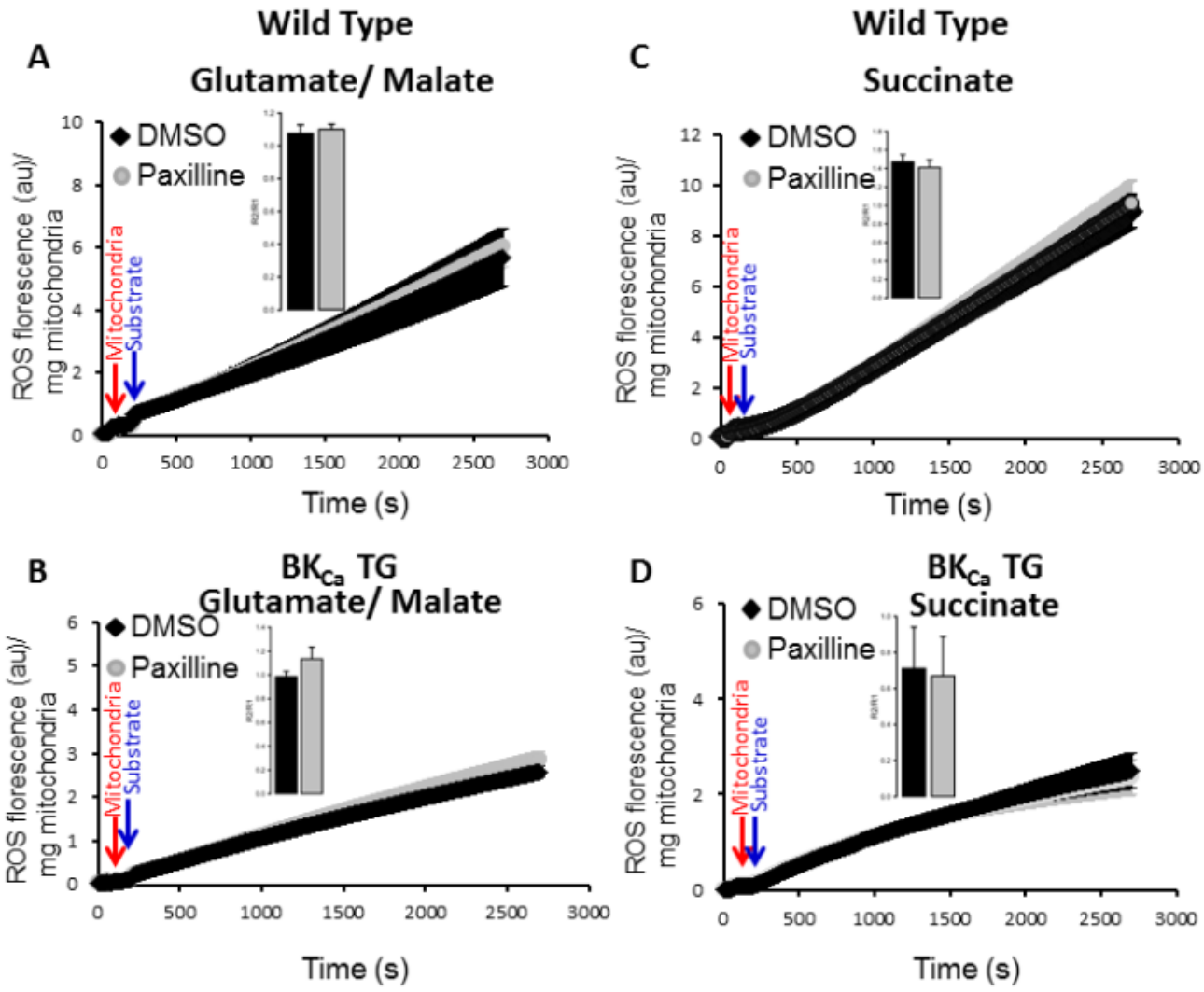

Fig 2. Measurement of Reactive Oxygen Species (ROS) in isolated mitochondria without IR. Mitochondria were isolated from wild type (A and C) and BK_{Ca} TG mice (B and D). Isolated mitochondria were added to the ROS buffer (red arrow) flowed by a specific substrate (blue). After 15 mins, DMSO or Paxilline were added. Rate (insets) of ROS generation after addition of DMSO/Paxilline was divided by ROS generated by mitochondria before addition of drugs. ROS was measured using amplex red. To measure ROS generated by complex I, glutamate/ malate was used as a substrate (A and B) and for complex II/III, succinate was used as a substrate (C and D). There was no significant difference in total ROS generated or rate of ROS generated (insets) by either complexes in wild type or BK_{Ca} TG mice with DMSO (black) or Paxilline (grey).

Figure 3

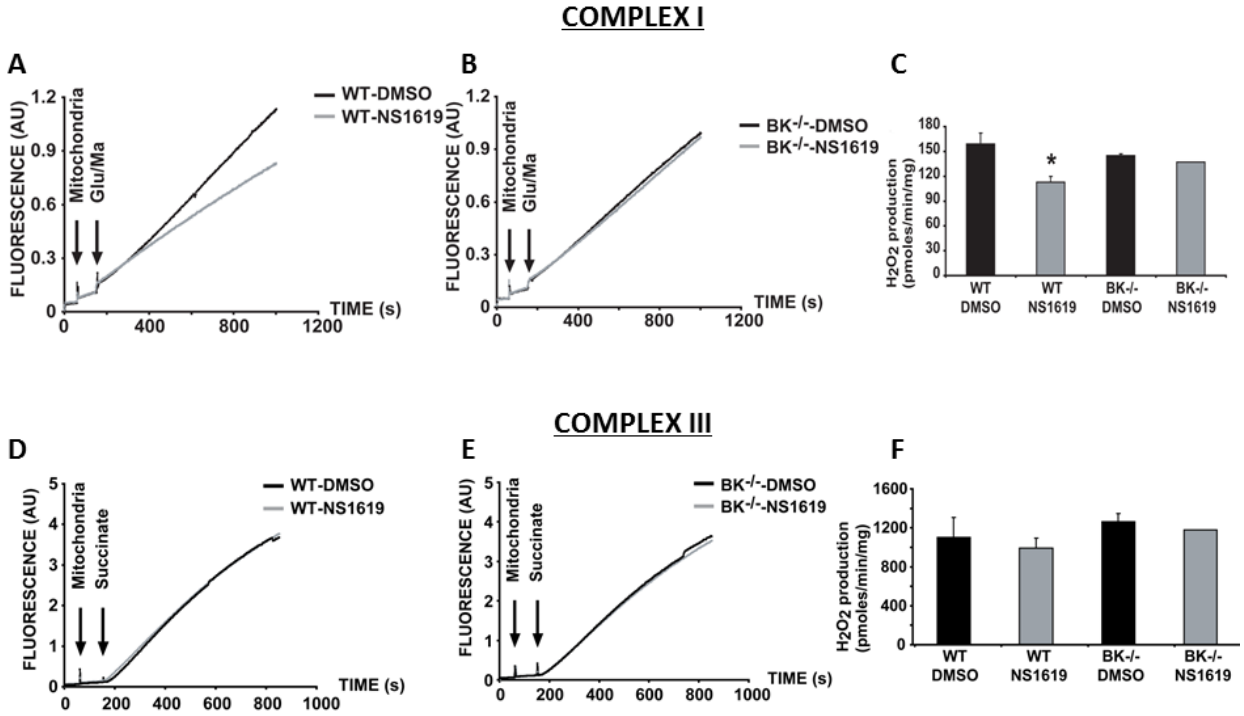

Fig 3. Measurement of Reactive Oxygen Species (ROS) in isolated mitochondria after IR. Mitochondria from wild type and *Kcnma1*$^{-/-}$ mice were isolated after 10 min of reperfusion following 18 min of global ischemia. Hearts were treated with 10 μm NS1619 before ischemia for 18 min. ROS was measured with amplex red for hearts pretreated with DMSO (vehicle control, black) or NS1619 (BK_{Ca}-agonist, grey). Opening of BK_{Ca} reduced ROS generated by complex I (A and C) in wild type but not in *Kcnma1*$^{-/-}$ (BK-/-) mice (B and C) with glutamate/malate as a substrate. There were significant differences observed in ROS generated by complex II/III (D, E, and F) with succinate as a substrate.

Research Project 12: Project Title and Purpose

Designing an Information System to Support Data-driven Decision-making in RTI – Response to intervention (RTI) is a framework used by schools to adhere to the Individuals with Disabilities Education Improvement Act (IDEA) and provide children the support they need to learn. RTI involves academic and behavioral intervention, tracking a student's progress, and frequently adjusting interventions as needed. The process of RTI should be data-driven, but schools lack the appropriate technology to manage and analyze data. The proposed work will design and validate an information system for real-time data collection, visualizations of aggregated data, and automatic identification of trends. The purpose of this work is to address health and educational disparities by ensuring RTI decisions are data-driven.

Anticipated Duration of Project

1/1/2015 – 12/31/2015

Project Overview

Our prior work defined the role of data in behavioral intervention by identifying four touchpoints during an iterative process. These touchpoints define when decision-makers need to share and analyze data: (1) assess needs for intervention, (2) determine appropriate intervention, (3) apply data-driven intervention, and (4) evaluate effects of intervention. We have developed a novel iPad-based information system, Lilypad, to support data-driven decision-making around these touchpoints.

Specific aims:
Aim 1: Add two types of features to Lilypad: visualizations (graphs) of data, and automated notifications based on detection of trends in the data.
Aim 2: Validate the design of the features added with input from users, to ensure the updated Lilypad system is ready for use.

Design activities will explore how these features can be designed to: increase how much time users spend reviewing the data; and help users interpret, and make decisions based on, data. This work will use the approaches of community-based participatory research and user-centered design. Participatory design workshops will be used to engage users in the design of features. The workshops will involve open discussion of user needs and barriers to data-driven decision making, and the review and critique of iterative designs we will present for feedback. Participants will evaluate mockups and provide input on system development based on the following open-ended questions: How does data inform placement decisions, and support referrals and transitions? What kind of automated notifications can alert you to behavioral trends, so you can more quickly adjust and calibrate behavioral interventions? How can we increase the amount of instruction time? How can we decrease the amount of time needed for data gathering and analysis? The ultimate goals of this work are to address health and educational disparities by ensuring RTI decisions are data-driven. To achieve this end, features will be designed to encourage the use of data by making it more accessible, and provide in the moment decision support by helping users identify trends in the data. Design efforts will focus on visualizations that enable the exploration of data over of time, and automated notifications to help users identify trends in the data.

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Expected Research Outcomes and Benefits

Data plays a large role in special education, where clinical services are integrated with educational services to address behavioral, emotional, and mental health needs that impact a child's opportunities to learn. Response to intervention (RTI) is an evidence-based framework that identifies at-risk students, before a special education referral so that the appropriate level of support can be provided. RTI involves tracking a child's progress, and frequently adjusting academic and behavioral interventions as needed. This process should be data-driven, but there are significant barriers to the use of data in decision-making. In this work we focus on three barriers to the use of data: 1) record keepers have difficulty capturing representative records, 2) decision makers do not have adequate access to aggregated records, and 3) the data is not represented in a way that aids interpretation and decision-making. Information systems have thus far not met these needs, causing a reliance on paper-based methods. Data is currently not represented in a way that aids interpretation and decision-making, in large part because most data is stored on paper. The ultimate goals of this work are to address health and educational disparities by ensuring RTI decisions are data-driven. Through a rigorous design process, we will develop a usable real-world system with the potential to impact the way RTI is implemented in special education. The technology we are developing is novel, and no decision support system like it currently exists. The technology itself, and the data-driven decision-making practices it will support, will be novel approaches to providing behavioral intervention. Future evaluations of our technology will provide evidence for individual outcomes as well as effects on health and educational disparities.

Summary of Research Completed

During the past six months, we have made progress on the design of Lilypad features to make decision-making more data-driven. The milestones for this period were participant recruitment, and workshop planning and preparation. We reached these milestones through our work with the special education team at Holly Heights Elementary School in Millville, NJ.

Through an existing relationship with Holly Heights Elementary School, we introduced the Lilypad project to the special education team and they expressed interest in using it in their classrooms. We obtained approval from school administrators, and recruited the special education team as participants in our study. Seven individuals make up the special education team and have signed consent forms for our study: two teachers, four classroom aides, and one supervisor. Our work has focused on these individuals and the activities in the two classrooms they work in.

We held three focus groups with the special education team to ask them questions about how they record and use behavioral data, and engage them in a discussion about how intervention decisions could be more data driven. In these focus groups, we also presented some design

concepts and visuals as probes (see attached figures) to obtain input on the design of the two types of features we are interested in: visualizations or graphs of data, and automated notifications based on detection of trends in the data.

We complemented focus group data with our own observations in the classroom setting. From April until June, two researchers on our team observed classroom activities twice a week to understand how data is recorded in the moment and what realistically happens with that data day to day in the classroom. After each observation session, at least two researchers on our team analyzed observation notes together using inductive thematic analysis and affinity diagramming. Once a week, these data were analyzed and findings were presented to the PI for feedback and guidance on further observation and data analysis. Observation continued until data saturation was reached and nothing new was being observed.

We have used the findings from this formative qualitative research to iterate on design ideas, following a user-centered design process. We have turned abstract ideas into concrete visuals that illustrate how each feature would look and work (see attached figures). These visuals represent a concrete milestone for this project for several reasons. First, they are the culmination of our research on existing data collection and decision-making practices, and the result of our analysis on how decision-making could be more data-driven. Second, they are how we have planned and prepared for workshops to be held in the second half of this work. Workshops will be based on these visuals, enabling us to discuss these concrete options for features and eliciting further feedback from participants in order to begin developing the technology.

Specific Aim 1 Progress:
We have generated a variety of concrete ideas represented by visual mockups of potential features. The attached figures show specific features that are ready to be handed to software developers on our team to be added to Lilypad. The remaining step before the development phase will be workshops to validate the features and elicit a final set of feedback from participants. The effectiveness of features we add to Lilypad will be dependent on this feedback, and we will be able to elicit more useful feedback with the visual mockups we have spent the past six months creating. Our work thus far has involved collecting and analyzing qualitative data to inform the design of these visual mockups.

The attached features include:

- How potential new features will look in the context of the existing Lilypad system, so that we can ensure usability and feasibility. Showing the context of use will also help workshop participants to provide useful feedback.
- Various types of bar graphs and line charts to determine which types of data representations will be most useful to aid with decision-making.
- Different ways to analyze the data, for example by date, by activity, by classroom, or by period of day.
- Automated notifications such as a badge system based on gold, silver, and bronze levels of performance. These performance levels are currently used and the design of the badge system was based on our research on current practices. Each level is defined based on quantifiable goals that must be reached and maintained over time. The badge system will

automate these calculations and notify relevant stakeholders as a way to make progress more clear and maintain consistency and communication across individuals involved in decision-making.

Specific Aim 2 Progress:
Our qualitative research has validated and refined each design idea. The attached figures represent the results of this research. Rather than abstract concepts or potential solutions that we came up with on our own, we engaged in participatory research to validate each feature from inception to visual design. We have elicited input from users through focus groups, and during observation we occasionally engaged them in discussions about what types of features would improve their data recording and analysis practices.

We also made Lilypad available for use for two weeks in our participants' classrooms, so they could experiment with it and evaluate the system as a whole during their daily work when possible. This gave participants a better sense of how they would use Lilypad in their daily work, enabling them to give us more accurate and detailed input into the design of new features, and the giving them the experience to validate, at least to some extent, that the new features would be feasible and usable in the context of their daily work.

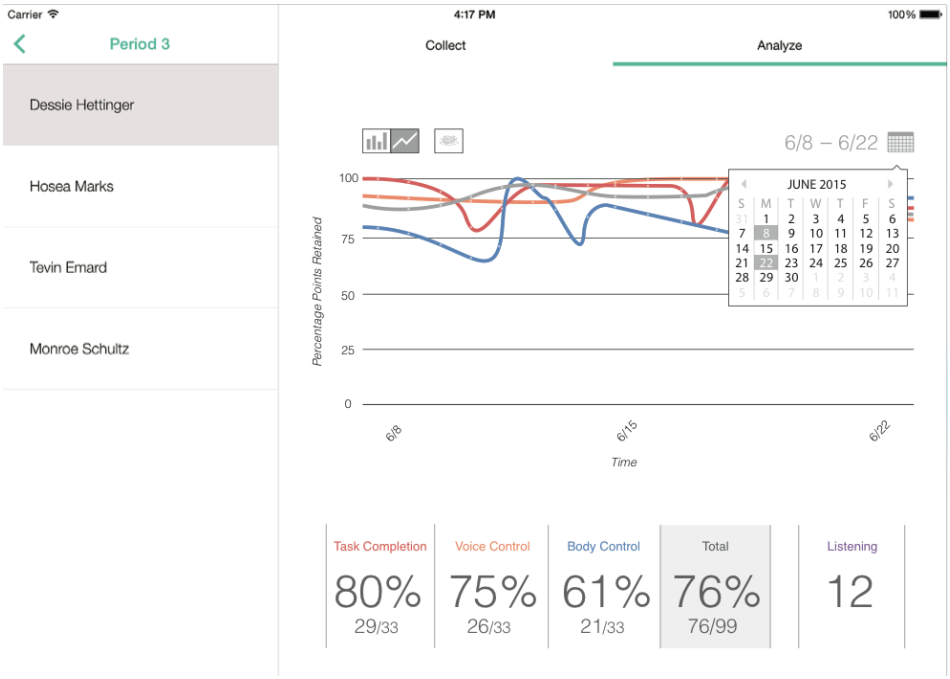

Figure 1: The section of the existing Lilypad interface that we are adding features to. Users can select the start date and end date for data they would like to analyze. Based on this framework, we have designed new visualizations of the data and automated notifications.

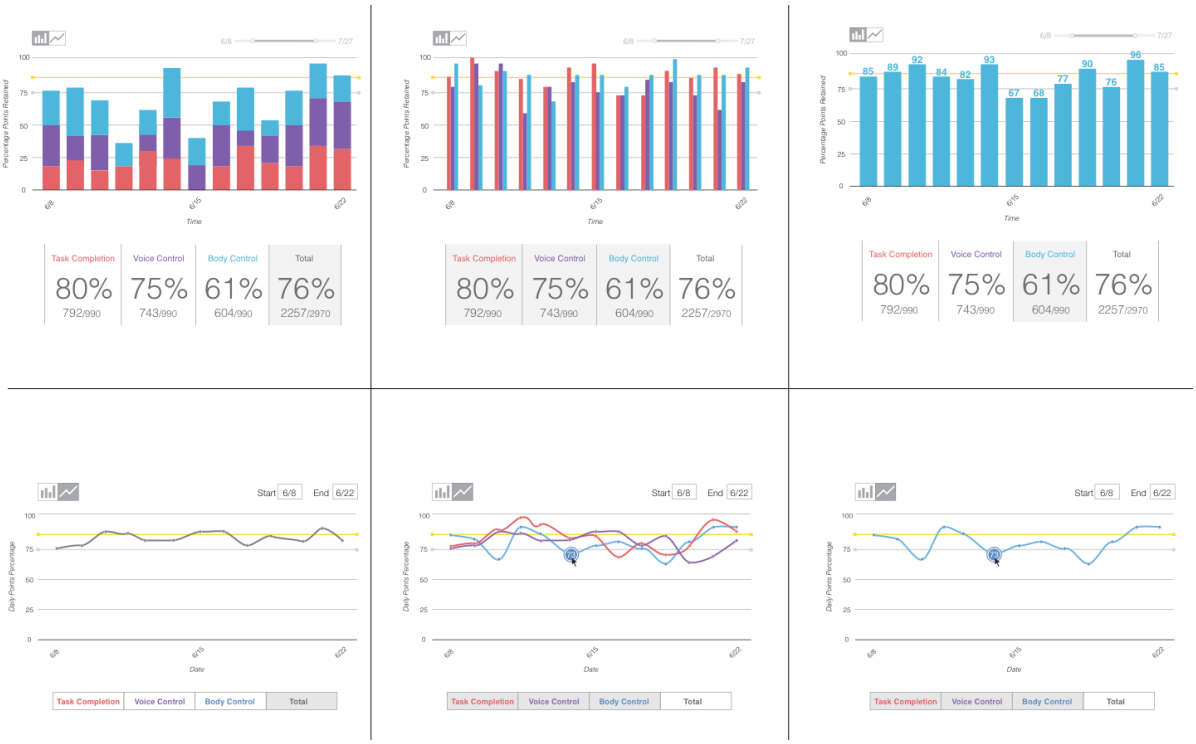

Figure 2: Graphs of the behavioral data, including different bar and line graphs, which we will show to participants during workshops to elicit input and finalize features to be added to Lilypad.

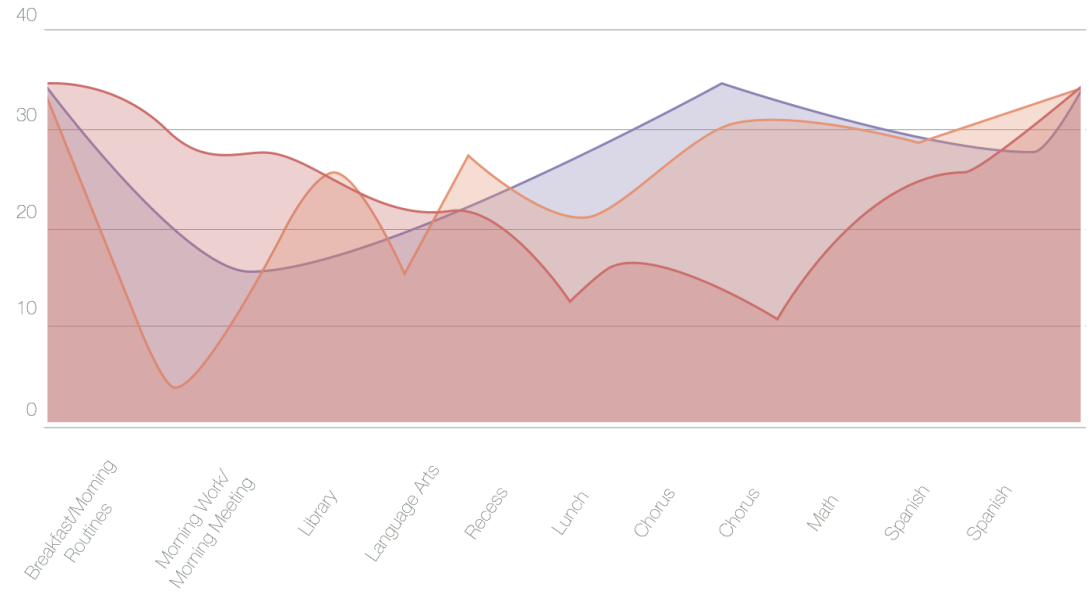

Figure 3: Other types of visualizations will enable analysis of behavioral data based on the activity during which the behaviors occurred, or other factors that may inform interpretation of data and resulting decision-making.

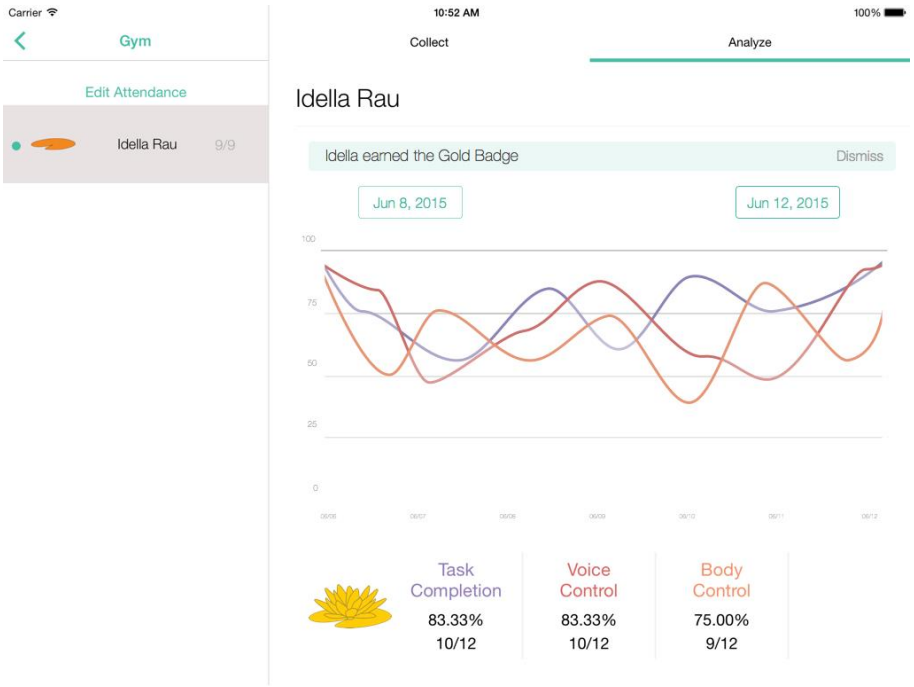

Figure 4: An example of one of the automated notifications, which will notify users and other relevant stakeholders that the student has reached a set goal (the gold badge, represented by a gold lilypad icon).

Research Project 13: Project Title and Purpose

Screening for Autism Spectrum Disorder in Child Care Settings – Early detection of Autism Spectrum Disorder (ASD) facilitates early intervention, which improves lifelong prognosis. Efforts focusing on universal screening in the medical home overlook an important setting with regular access to young children: childcare settings. The current project will used a mixed-method design to pilot ASD-specific screening in five childcare centers in the Promise Zone, an underserved neighborhood in West Philadelphia to (1) demonstrate feasibility and acceptability of screening in childcare centers, and (2) gather empirical data on the validity of ASD screening in childcare.

Anticipated Duration of Project

1/1/2015 – 12/31/2015

Project Overview

Early detection of Autism Spectrum Disorder (ASD) facilitates early intervention, which improves lifelong prognosis. Efforts have focused on universal screening in the medical home. However, research is lacking on whether screening can be effective in non-medical settings, such as childcare centers. Engagement of early care and education providers (ECEPs) in toddler

screening will address this research gap. Therefore, the objectives of this pilot study are: (1) to demonstrate feasibility and acceptability of screening in childcare centers – important empirical measures of implementation science – with particular emphasis on screening underserved children, and (2) to examine the performance of a widely used screening tool in a novel setting: child care centers. The specific aims of this study are to:

(1) Demonstrate that the Modified Checklist for Autism in Toddlers, Revised, with Follow-Up (M-CHAT-R/F) can be completed both by parents and ECEPs in the child care setting (feasibility). Hypothesis: Both parents and ECEPs will successfully complete the M-CHAT-R/F on children within the eligible age range; at least 80% eligible toddlers will be screened.
(2) Examine satisfaction with screening. Satisfaction questionnaires will demonstrate that both parents and ECEPs find screening to be feasible and acceptable. Hypothesis: Ratings for ease of use will be very high, ratings for needing help to complete forms will be very low.
(3) Compare results based on informant (parent vs. ECEP). Hypothesis: Agreement in ASD risk will be very high between parents and teachers.
(4) Demonstrate that knowledge about developmental milestones and screening will increase as a results of the ECEP training and the implementation of the screening protocol in the childcare setting. Hypothesis: post-test measures of knowledge will demonstrate significantly more knowledge about milestones and screening compared to pre-test measures.
(5) Compare preliminary psychometric properties (i.e., sensitivity, specificity) for screening in childcare settings to published psychometrics for screening in medical settings. Hypothesis: sensitivity and specificity will be comparable when screening in this novel setting.

To meet these aims, a mixed method design will pilot toddler ASD screening on at least 200 children in five childcare settings located in the Philadelphia Promise Zone. Mixed method designs integrate both qualitative and quantitative research; this is an important approach for a pilot research study where the quantitative data may not have adequate power to run sophisticated statistical analyses. Instead, the combination of qualitative and quantitative methods allows for more in-depth examination of small samples that are standard for pilot studies. Qualitative data from focus groups will be collected at project start to address feasibility and acceptability of screening. Several forms of quantitative data will be collected in this research study. Pre- and post- measures of ECEPs' knowledge of developmental milestones and beliefs about ASD screening will be directly compared to demonstrate knowledge gain from training and screening. Prospective data on screening utilization will also be collected, including number of children screened, frequency of discussing concerns with parents, and rate of referrals for early intervention. These data will allow quantitative measurement of feasibility, and demonstrate that screening will be completed on more than 80% of eligible toddlers. The screening tool is the M-CHAT-R/F, validated in 16,000 toddlers and demonstrated to have strong sensitivity and specificity to detect ASD. Children who screen positive will be offered a diagnostic evaluation at the AJ Drexel Autism Institute at no cost. Evaluation of screen positive cases will allow preliminary calculations of the psychometric properties of M-CHAT-R/F screening when implemented in a childcare setting. These values will be compared to published psychometric properties when M-CHAT-R/F is implemented in pediatric medical settings.

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Expected Research Outcomes and Benefits

Children in childcare will be screened for ASD to achieve the following research outcomes:

1) Psychometric properties (e.g., sensitivity) will be calculated and compared to published validity of ASD screening in pediatric settings. Chi-square analyses will indicate that validity is not significantly different based on setting (childcare vs. pediatrician), demonstrating that universal screening will be more successful if disseminated across diverse community settings.
2) Focus group findings will demonstrate feasibility and acceptability of screening in childcare settings. Themes from focus groups will be analyzed and reported in deidentified format. Themes also may impact ECEP training and development of future training materials.
3) ASD screening to date has emphasized parent-report tools, and little research has examined results across different informants. Comparison of parent- and ECEP-report will indicate that both parents and ECEPs can successfully complete screening questionnaires about ASD risk. Data regarding agreement in screening results across ECEPs and parents will inform future studies about whether informant-specific instructions or item wording might improve validity.
4) These outcomes will lead to a clearer understanding of how to conduct ASD training for non-health professionals, as well as how to conduct ASD screening in settings outside of the medical home. Our ECEP training methods, which include measuring pre- and post-questionnaires about developmental milestones, informational videos, and practice situational role play will help to increase self-efficacy and knowledge, in addition to informing future training opportunities for ECEP's and other community based providers who work with young children. By working with professionals in daycare settings, we are helping to bridge the gap between early diagnosis and intervention and disadvantaged children who may not regularly be able to visit the doctor for ASD screening. This project will also help to improve the quality of life of children affected by ASD, by providing evaluation services to those that screen positive, at no cost. It is also anticipated that enhanced understanding of ASD knowledge and the process of screening will increase awareness among parents, who will be more likely to follow through with referrals and support.

Summary of Research Completed

Specific Aim 1 Progress: No progress to report during this reporting period
Specific Aim 2 Progress: No progress to report during this reporting period
Specific Aim 3 Progress: No progress to report during this reporting period
Specific Aim 4 Progress:
Project personnel attended several community events in order to increase awareness about the screening study, and to connect with potential childcare sites. These events included monthly community dinners at the Dornsife Center, Delaware Valley Association for the Education of Young Children (DVAEYC) member meetings, and the West Philadelphia Community Center Community Expo.

Preparation for the focus groups included developing a targeted set of seven questions, with multiple probe options, for use in parent and teacher focus groups. Questions were tailored to each focus group individually. All participants consented to the study and to audiotaping Transcripts were created based on audio recordings, and entered in NVivo for theme analysis. The 1-hour parent focus group was completed in March 2015. This group included nine parents or grandparents who lived within the Promise Zone of Philadelphia and had young children in their home. The age of parents/grandparents ranged from 20-56 years old (*mean*=42, *SD*=12.8). The age of children in their homes was 24-60 months (*mean*=48, *SD* =13.6). The participants were predominately African American (88.89%), and 44.4% of participants held a degree higher than a high school diploma. Please see Table 1 for additional demographics. Approximately one third of participants voiced familiarity and shared facts about autism with the group.

Parents were asked a series of questions, including questions about their current methods of childcare, and what they felt were the biggest strengths and weaknesses of their current childcare. The majority of parents (77.8%) placed their child in childcare, and felt socialization and learning foundational skills such as sharing were benefits to this method. One participant did not have their child enrolled outside of family-based care, and one participant did not specify.

In addition to these questions, parents were probed about their feelings and beliefs about their childcare provider's knowledge and abilities to perform assessments on their child's developmental abilities. The majority of participants (77%) voiced some type of concern at least one time during the focus group about their childcare provider's abilities and training and felt they were not qualified to make those types of conclusions. Two participants felt that if their teacher provided them with feedback about their child's development, they would immediately get a second opinion. Lack of training and familiarity, and lack of a relationship with their children were all common concerns with childcare providers among participants. Two participants had children that received some type of early intervention services, but this need was brought to their attention by their pediatrician, not their childcare provider.

The 1-hour teacher/childcare center director focus group was completed in June 2015. This group included seven ECEPs and childcare center directors. The participants ranged from 36-61 years old, were predominately African American (85.7%), and 57.1% of participants held an

advanced degree. Please see Table 2 for additional demographics. All participants consented to the study and to audiotaping. Results were entered into NVivo software to identify themes. Preliminary results indicate that ECEPs identified building a relationship with parents as critical for talking about development and risk for delay (43%). Participants also were enthusiastic about the role of childcare providers in developmental monitoring and screening, and endorsed use of different methods of continuous monitoring for children in their care (57% informal, 28% Ages & Stages questionnaire, 14% eclectic approaches).

Next, the Early Child Education Provider (ECEP) training was developed. A review of literature and existing research, as well as reliable information from larger established organizations were all used while preparing the teacher training. The research coordinator and principal investigator collaborated to develop a streamlined presentation that addressed background information about autism spectrum disorder (ASD), including symptoms, prevalence, and disparities in early identification; video examples of risk signs for ASD that can be observed in childcare settings; procedural details about the screening study; an interactive activity addressing observations of children in current or past classes that represent both typical and atypical social, communication, and play behaviors as well as presentation of restricted, repetitive, or stereotyped behaviors; and discussion of how to talk with parents about risk for developmental delay. Video examples were extracted from www.wellchildlens.com with the owner's permission. ECEPs were informed that additional videos are available at the above website for those who are interested in learning more. A manual was also put together for ECEPs to reference during the study. The manual included screen shots of the web based system, and step-by-step directions on how to complete the screening from both teacher and parent roles.

Eight childcare centers have agreed to participate in the study. Four childcare centers have completed ECEP training. During the completed trainings, 20 ECEPs consented to the study, and completed the pre-questionnaire about knowledge and beliefs regarding screening for ASD, and the role of ECEPs in the early detection of ASD. See Table 3 for ECEP Demographics. Most teachers reported that they monitored development in their classrooms (n=18), have talked to parents about concerns of their child's development (n=16), and some have suggested parents consult with experts about their child's development (n=11). Enrolled ECEPs were asked to prioritize 7 topics pertaining to child development and safety on a 1-5 scale with 1 being lowest priority and 5 being highest priority. Many ECEPs ranked all topics as equal to highest priority. The highest ranking priorities were safe storage of household chemicals or weapons (mean rating 4.80) and developmental screening including autism (mean rating 4.65). Next priority was car seats and transportation (mean rating 4.55). See Table 4 for results regarding the role of childcare providers in developmental monitoring and screening. Overall, results indicated a moderately high level of importance attributed to monitoring and screening for developmental delay. Most ECEPs reported "neutral" to "very likely" when asked if they would use questionnaires and lists of developmental milestones in their care of toddlers. The highest endorsement was for 'developmental milestones which teachers review with parents', and second highest were for 'questionnaires from the center or child development organizations completed by the teachers'.

In all four completed trainings, the ECEPs were engaged; they asked thoughtful questions, suggested example cases to discuss during the interactive activity, and had comments or

suggestions regarding deployment of screening in their centers. However, at one center, ECEPs expressed concern that many parents whose children attend their center are reluctant to engage in research. The group brainstormed approaches that may help encourage parents to participate without any pressure or coercion. Approaches discussed include, a parent information session is being planned for parents who want to hear more about the study. In addition, the research coordinator and additional student personnel will be scheduling several hours each week to visit the enrolled centers, centered on the busiest drop off and pick up times, in order to assist parents in completing the screening. All centers anticipate beginning screening during July 2015.

Some centers have fairly low enrollment of children in the target age range (15-36 months), and others report that not all families are willing to participate in research. As a result, two modifications are in progress. First, we have decided to increase the upper age limit to 47.99 months. This is consistent with published literature using the M-CHAT to screen preschool children (e.g., Janvier et al., 2015; Snow & Lecavalier, 2008). Second, if the target of five childcare centers does not lead to screening of approximately 200 children, we will increase enrollment to additional childcare centers. The consultant from the Delaware Valley Association for the Education of Young Children (DVAEYC) will begin inviting additional childcare centers to participate next month. It is anticipated that the full sample will be collected prior to the end of the study.

Specific Aim 5 Progress: No progress to report during this reporting period

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Table 1. Parent/Family Focus Group Participant Demographics (n=9)

Relationship to Child			
Mother		56%	
Father		11%	
Grandmother		33%	
Parent Martial Status			
Married		22%	
Single		56%	
No Response		22%	
Parent Education Level			
No degree		11%	
GED/HS		45%	
Vocation School		11%	
Associates Degree		22%	
Advanced Degree (Masters, PhD)		11%	
Parent Income Level (monthly)			
<500		11%	
501-2000		56%	
2001-4000		22%	
4001-6000		11%	
	Mean	**SD**	**Range**
Parent Age	42 yrs	12.8	20 - 56 years
Child Age	48 mos	13.6	24 - 60 mos

Table 2. Demographics of ECEPs in Focus Group (n=7)

Sex	***N* (%)**		
Male	0 (0%)		
Female	7 (100%)		
Race/Ethnicity			
African-American	6 (85.7%)		
Caucasian	1 (14.2%)		
Education			
High School/GED	1 (14.2%)		
Associate's Degree	1 (14.2%)		
Bachelor's Degree	1 (14.2%)		
Advanced Degree	4 (57.1%)		
	Mean	**SD**	**Range**
ECEP Age	46.5	10.50	36-61
Years as ECEP	17	7.75	5-25

Table 3. Demographics of ECEPs at Training (n=20)

Sex	***N* (%)**		
Male	0 (0%)		
Female	20 (100%)		
Race/Ethnicity			
African-American	10 (58.8%)		
Caucasian	5 (29.4%)		
Asian	1 (5.8%)		
Multi-Racial	2 (11.7%)		
Education			
High School/GED	8 (47.0%)		
Associate's Degree	6 (35.3%)		
Bachelor's Degree	5 (29.4%)		
Advanced Degree	1 (5.8%)		
	Mean	**SD**	**Range**
ECEP Age	36.45	11.39	20-60
Years as ECEP	9.55	7.38	0-25
# Children in Classroom	11.06	5.43	3-22

Table 4. Pre-Study opinions about the role of childcare providers in developmental monitoring and screening (1=Strongly Disagree, 2=Disagree, 3=Neutral, 4=Agree, 5=Strongly Agree); n=17 ECEPs

Item	Mean	SD	Range
I have the experience to identify most children with developmental delays or signs of possible autism	3.68	1.03	2-5
Using parental concern about a child's development and/or behavior is important	4.58	0.51	4-5
Using classroom observations about a child's development is important	4.60	0.50	4-5
If I identify developmental delays or signs of possible autism in a child, I feel confident in how to talk with the child's parents about the delays and/or signs of autism	3.50	1.0	2-5
Using broad developmental screening tools for all children in childcare is important	4.0	0.92	2-5
Using autism-specific screening tools for all children in childcare is important	3.9	0.97	2-5
Using broad developmental screening tools in childcare when I am concerned a child has a developmental delay is important	4.05	1.05	1-5
Using autism-specific screening tools in childcare when I am concerned a child has a developmental delay is important	4.10	1.07	1-5
If I identify developmental delays or signs of possible autism in a child, I feel confident in how to care for the child, including how to refer the family for additional services	3.8	0.86	2-5